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Elucidating the role of ascorbic acid in flowering and pathogen defense in Arabidopsis thaliana

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Elucidating the Role of Ascorbic Acid in Flowering and Pathogen Defense in *Arabidopsis thaliana*

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ABSTRACT

Elucidating the Role of Ascorbic Acid in Flowering and Pathogen Defense in *Arabidopsis thaliana*

Madhumati Mukherjee

L-ascorbic acid (AA) protects plants against abiotic stress. Previous studies suggested that this antioxidant is also involved in the control of flowering and enhanced disease resistance. This study aimed to elucidate the mechanisms through which AA deficiency promotes flowering and pathogen resistance.

To decipher how AA influences flowering time, four AA-deficient *Arabidopsis thaliana* mutants *vtc1-1*, *vtc2-1*, *vtc3-1* and *vtc4-1* were grown under short and long days, respectively. These mutants flowered and senesced before the wild type irrespective of the photoperiod, a response that cannot simply be attributed to slightly elevated oxidative stress in the mutants. Genetic analyses demonstrated that various photoperiodic and autonomous flowering pathway mutants are epistatic to the *vtc1-1* mutant. Our genetic transcript analyses suggest that AA acts upstream of the photoperiodic and autonomous pathways. However, a specific pathway in which AA acts could not be pinpointed. This suggests that AA plays a more general role in adjusting flowering time depending on environmental conditions.

The AA-deficient *Arabidopsis vtc1-1* mutant was previously shown to exhibit increased resistance to the virulent bacterium *Pseudomonas syringae*. This response correlates with heightened levels of salicylic acid (SA), which induces antimicrobial pathogenesis-related (*PR*) proteins. To determine if SA-mediated enhanced disease resistance is a general phenomenon of AA deficiency, to elucidate the signal that stimulates SA synthesis, and to identify the biosynthetic pathway through which SA accumulates, the four AA-deficient *vtc1-1*, *vtc2-1*, *vtc3-1*, and *vtc4-1* mutants were studied. Double mutants with a defect in the AA-biosynthetic gene *VTC1* and the SA signaling pathway genes *PAD4*, *EDS5*, and *NPR1*, respectively, were generated and studied. All *vtc* mutants were more resistant to *P. syringae* than the wild type. With the exception of *vtc4-1*, this correlated with constitutively upregulated H_2O_2, SA and mRNA levels of *PR* genes. Double mutants exhibited decreased SA levels and enhanced susceptibility to *P. syringae* compared to the wild type, suggesting that *vtc1-1* requires functional PAD4, EDS5 and NPR1 for SA biosynthesis and pathogen resistance. This work suggests that AA deficiency causes constitutive priming through a buildup of H_2O_2 that stimulates SA accumulation conferring enhanced disease resistance in *vtc1-1*, *vtc2-1*, and *vtc3-1*, whereas *vtc4-1* might activate defense responses after infection.

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1.1INTRODUCTION

1.1.1. Functions of ascorbic acid as an antioxidant and cofactor

L-ascorbic acid (AA, vitamin C) is a multifaceted molecule with diverse physiological functions in plants and animals. It serves as an antioxidant and enzyme cofactor and is an important vitamin for animals that have lost the ability to synthesize AA. In animals, insufficient intake of AA leads to collagen deficiency, leading to scurvy (Smirnoff, 1996).

The functions of AA in the detoxification of reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), are well characterized (Smirnoff, 2000). In plants that grow under optimal conditions, cellular ROS production is low. However, the formation of ROS increases under adverse environmental conditions, including drought, salinity, air pollutants or pathogen attack (Padh, 1990). Since ROS can cause cellular damage, their production and detoxification must be controlled by protective mechanisms. This is accomplished by antioxidant enzymes, including superoxide dismutase and ascorbate peroxidase, which detoxifies H_2O_2 to water using AA as a reductant, AA-regenerating enzymes, such as monodehydroascorbate reductase and dehydroascorbate reductase, and other antioxidants, primarily glutathione (Niyogi, 2000; Mittler, 2002). Besides being a co-substrate for AA peroxidase, AA is also a cofactor for 2-oxoacid-dependent dioxygenases that are involved in the biosynthesis of the plant hormones abscisic acid, gibberellin, and ethylene (Arrigoni and De Tullio, 2000; 2002). However, ROS are also involved in signal transduction pathways controlling pathogen defense responses and programmed cell death, which correlates with a suppression of ROS detoxification mechanisms (Hammond-Kosack and Jones, 1996; Desikan et al., 2001; Neill et al., 2002).

Research over the past decade has suggested that AA has functions in addition to its antioxidant properties. These scientific advances would not have been possible without the isolation of AA-deficient *Arabidopsis thaliana vtc* mutants.

1.1.2. *Arabidopsis thaliana* vitamin C-deficient (*vtc*) mutants

Four vitamin C-deficient (*vtc*) mutants, *vtc1*, *vtc2*, *vtc3*, and *vtc4*, of the model plant *Arabidopsis thaliana* were originally isolated by Conklin and coworkers (Conklin et al., 2000). The genes that are defective in these mutants encode enzymes that catalyze steps in the predominant AA biosynthetic pathway in which glucose is converted to AA via D-mannose and L-galactose intermediates (Fig 1-1; Wheeler et al., 1998). The *VTC1* gene encodes a GDP-mannose pyrophosphorylase. The *vtc1-1* mutant contains a point mutation in *VTC1*, resulting in decreased enzyme activity and a 70% lower AA content compared to the wild type (Conklin et al., 1999). A point mutation in the *vtc2-1* mutant causes AA levels that are diminished by 70%-80% of the wild-type AA content (Conklin et al., 2000; Jander et al., 2002). VTC2 has GDP-L-galactose phosphorylase (Laing et al., 2007; Linster et al., 2007; Smirnoff et al., 2007) and L-galactose 1-P guanylyl-transferase activity (Laing et al., 2007; Linster et al., 2007). However, VTC2 GDP-L-galactose phosphorylase activity is predominant and a second enzyme, encoded by *VTC5* with high sequence identity to *VTC2*, also has GDP-L-galactose phosphorylase activity. Both VTC2 and VTC5 are required for AA synthesis and seedling viability (Dowdle et al., 2007; Linster et al., 2008). The *VTC3* gene has not yet been identified. *VTC4* encodes an L-galactose-1-phosphate phosphatase (Laing et al., 2004; Conklin et al., 2006). A mutation in this gene causes a 50% lower AA content in *vtc4-1* mutants compared to the wild type. Physiological characterization of these *vtc* mutants revealed that they are more susceptible to oxidative stress, as expected (Conklin et al., 1997; 2000). However, the *vtc* mutants also exhibit an array of pleiotropic developmental and metabolic defects, including altered flowering and senescence (Barth et al., 2004; Conklin and Barth, 2004; Pavet et al., 2005; Kotchoni et al., 2009), increased resistance to virulent pathogens (Pastori et al., 2003; Barth et al., 2004; Pavet et al., 2005), elevated levels of abscisic acid (Pastori et al., 2003; Kotchoni and Barth, unpublished data), salicylic acid (Barth et al., 2004), and a decreased content of gibberellic acid (Kiddle, 2004; Foyer et al., 2007). Since this project aimed to understand how alterations in the AA content influence flowering time and pathogen defense response, the following paragraphs introduce the current state of knowledge of these two processes in *Arabidopsis thaliana*.

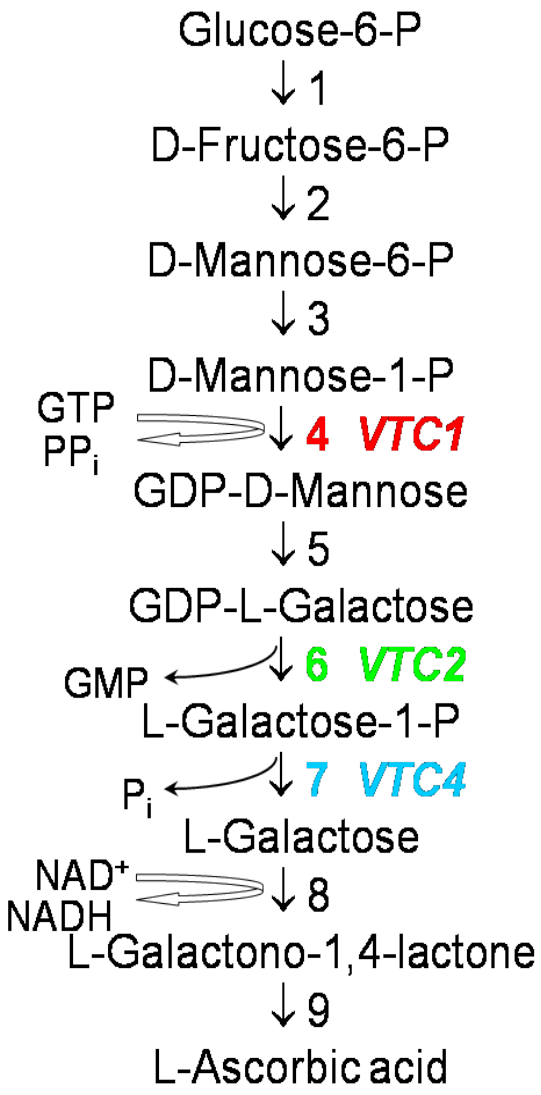

Figure 1-1: **Simplified representation of the D-mannose/L-galactose L-ascorbic acid biosynthetic pathway in higher plants**. Enzymes are (1) phosphoglucose isomearse, (2) phosphomannose isomerase (3), phosphomannose mutase (*PMM*), (4) GDP-mannose pyrophosphorylase (*VTC1*), (5) GDP-mannose-3',5'-epimerase, (6) GDP-L-galactose phosphorylase (*VTC2*/*VTC5*), (7) L-galacotse-1-phosphate phosphatase (*VTC4*), (8) L-galactose dehydrogenase, (9) L-galactono-1,4-lactone dehydrogenase. Adapted from Wheeler et al, 1998; Conklin et al. 1999; Dowdle et al. 2007.

1.1.3. Regulation of flowering in *Arabidopsis thaliana* and the role of ascorbic acid in flowering

Flowering time is controlled by external and internal factors that are integrated in a complex gene regulatory network that ensures the expression of flowering time genes, resulting in flower formation (Corbesier and Coupland, 2005). Environmental factors regulating flowering include day length, light, and temperature. The plant hormone gibberellin (GA) is an important internal factor that controls flowering. We therefore differentiate four flowering pathways in the facultative long-day plant *Arabidopsis thaliana*; the photoperiodic, vernalization, autonomous, and GA pathways.

One of the most important environmental factors that affect floral transition is the change in day length (photoperiod). A role of photoperiod was originally proposed by Tournois and Klebs (Tournois, 1912; Klebs, 1913). In the 1920s, Garner and Allard (Garner and Allard, 1920, 1923) were the first who discovered that flowering and other developmental responses could be controlled by exposure to short day (SD) or long day (LD) depending on the plant species. They introduced the terms photoperiod, which defines the recurring duration of daily light and dark periods, and photoperiodism, which defines the responses to photoperiod. Numerous studies have been devoted to elucidate the molecular mechanisms of the photoperiodic flowering pathway (Imaizumi and Kay, 2006; Kobayashi and Weigel, 2007). This pathway, which consists of a circadian clock and a circadian-regulated day-length measurement mechanism, promotes flowering specifically under LD. The *Arabidopsis thaliana* circadian clock is set by light signals that are perceived by red and far-red light receptors (phytochromes; primarily PHYA and PHYB), and by blue-light receptors (cryptochromes; CRY1 and CRY2). The core oscillator is composed of proteins, including CCA1 (CIRCADIAN CLOCK ASSOCIATED 1), LHY (LATE ELONGATED HYPOCOTYL) and TOC1 (TIMING OF CAB EXPRESSION 1), that are regulated in a negative-feedback loop (Schaffer et al., 1998; Strayer et al., 2000; Alabadi et al., 2001). The circadian clock takes part in the day-length measurement mechanism that is determined by the regulation of *CONSTANS* (*CO*) gene expression and the light-regulation of CO protein stability and activity (Hayama and Coupland, 2004). Induction of flowering in LD is regulated by the gene sequence *GIGANTEA* (*GI*)*–CO–FT*. In leaves, *GI* activates *CO* transcription regardless of

photoperiod (Fowler et al., 1999). *CO* transcription is regulated by a number of factors, including FKF1, GI, and CDF1 (Fowler et al., 1999; Suárez-López et al., 2001; Imaizumi et al., 2003; Chen and Ni, 2006; Sawa et al., 2007). CO protein is stabilized by PHYA, CRY1, and CRY2, whereas PHYB promotes degradation of CO (Valverde et al., 2004). Recently, a role of CRY signaling in suppressing COP1-mediated degradation of CO in the dark has also been reported (Jang et al., 2008; Liu et al., 2008). CO induces the expression of the floral integrator *FLOWERING LOCUS T* (*FT*) (Putterill et al., 2004). Finally, FT protein moves through the phloem to the shoot apex where FT interacts with the FD transcription factor to induce expression of the floral identity gene *APETALA1*, which requires the transcription factor *LFY* (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007). Expression of *LFY* is upregulated by FT (Blazquez, 2005).

Vernalization, i.e. low temperature treatment, is an environmental factor that promotes flowering (Martinez-Zapater et al., 1994) by repression of the floral repressor FLC (FLOWERING LOCUS C). Through the action of various genes, including *FCA* (*FLOWERING TIME CONTROL PROTEIN ALPHA, BETA, GAMMA, DELTA*), *FLC* is also repressed by the autonomous pathway that induces flowering independently of environmental cues (Quesada et al., 2003; Boss et al., 2004). Finally, GA promotes flowering of *Arabidopsis* under SD (Wilson et al., 1992; Blazquez et al., 1998; Eriksson et al., 2006). These four flowering pathways converge on the transcriptional regulation of *FT* and *SOC1 (SUPPRESSOR OF CONSTANS 1),* which promote *LFY* expression to confer floral identity on developing floral primordia (Corbesier and Coupland, 2005).

Early studies have suggested a role of AA in the control of flowering (Chinoy et al., 1957; Hillman, 1962; Bharti and Garg, 1970). The *vtc1* mutant was reported to flower and senesce before the wild type when grown under LD (Barth et al., 2004; Conklin and Barth, 2004). Interestingly, this mutant and the *vtc2* mutant were shown to exhibit delayed flowering and senescence when grown under SD (Pavet et al., 2005). Further support for a role of AA in the regulation of flowering time comes from the observation that artificially increasing the AA content delays flowering in LD-grown *Arabidopsis* (Attolico and De Tullio, 2006) and *Brassica rapa* (Daniela and De Tullio, 2007), but induced flowering in *B. rapa* when grown under SD (Daniela and De Tullio, 2007). Based on these reports and current knowledge about mechanisms regulating flowering,

several hypotheses were formulated to explain the contrasting flowering phenotypes when AA levels are altered (Barth et al., 2006). However, it was recently reported that all four *vtc* mutants, except *vtc2-1*, exhibit an early flowering phenotype under both conditions, suggesting that AA deficiency causes this phenomenon irrespective of the photoperiod (Kotchoni et al., 2009).

1.1.4. The *Arabidopsis* salicylic acid pathogen defense pathway and the role of ascorbic acid in disease resistance

The salicylic acid (SA) defense signaling pathway is activated by avirulent pathogens, causing the hypersensitive response (a form of programmed cell death), and by virulent pathogens, including *Pseudomonas syringae* and *Hyaloperonospora parasitica* (Glazebrook, 2001; Nimchuk et al., 2003; Glazebrook, 2005). In response to pathogen attack, SA levels rise, resulting in activation of pathogenesis-related (*PR*) genes, which have antimicrobial activity (Fig 1-2). Salicylic acid accumulation requires interaction of PAD4 (PHYTOALEXIN DEFICIENT 4) and EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) (Zhou et al., 1998; Falk et al., 1999; Feys et al., 2001). Both genes encode triacyl-glycerol lipases. However, lipase activity does not appear to be important for the function of either protein (Falk et al., 1999; Jirage et al., 1999; Feys et al., 2001). PAD4 and EDS1 are required for the expression of EDS5, which encodes a MATE family transporter that presumably transports intermediates for SA biosynthesis (Nawrath et al., 2002). Therefore, EDS5 is essential for the accumulation of SA upon pathogen attack (Nawrath and Metraux, 1999). The *SID2* gene encodes isochorismate synthase (ICS1), an enzyme in the SA biosynthesis pathway. Mutants with a defect in *SID2* contain strongly reduced levels of SA, suggesting that in *Arabidopsis* this hormone is predominantly produced from isochorismate and not phenylalanine, as previously thought (Yalpani et al., 1993; Leon et al., 1995; Mauch-Mani and Slusarenko, 1996; Coquoz et al., 1998; Ribnicky et al., 1998; Wildermuth et al., 2001). Increasing SA levels promote dissociation of NPR1 (NONEXPRESSER OF PR1) oligomers into monomers in the cyotosol through reduction of disulfide bonds between the NPR1 monomers, which then enter the nucleus. There, the monomers interact with TGA transcription factors to

promote expression of PR1 and, presumably, other SA-regulated genes (Fig 1-3; Zhang et al., 1999; Kinkema et al., 2000; Fan and Dong, 2002; Despres et al., 2003; Johnson et al., 2003; Mou et al., 2003; Tada et al., 2008). After localized foliar infections by diverse pathogens, plants often develop whole plant immunity, known as systemic acquired resistance (SAR; Durrant and Dong, 2004). In this process, which, in addition to pathogen attack, may be induced by treatment with certain natural or synthetic compounds (Conrath et al., 2006; Beckers and Conrath, 2007), distal (systemic) leaves become primed to activate a stronger defense response upon secondary infection (Ryals et al., 1996). This type of resistance also depends on the accumulation of SA.

Increased pathogen resistance was first described in *vtc1-1* and *vtc2-1*, which support less growth of the virulent bacterial pathogen *Pseudomonas syringae* pv. *maculicola* ES4326, the cause of leaf spot disease, and the virulent oomycete *Hyaloperonospora parasictica* pv. *Noco*, which causes downy mildew (Barth et al., 2004; Pavet et al., 2005). The enhanced pathogen resistance correlates with higher transcript and protein levels of PR proteins PR1 and PR5, increased levels of SA, and premature senescence (Barth et al., 2004). It was suggested that AA deficiency promotes expression of genes in the SA defense signaling pathway and that premature senescence contributes to this enhanced pathogen resistance (Kus et al., 2002). Pavet et al. (2005) investigated whether AA deficiency activates plant innate defense responses via redox mechanisms. The authors reported that the regulatory protein NPR1 moves into the nucleus where it activates the expression of *PR* genes (Mou et al., 2003) via TGA transcription factors (Despres et al., 2003). The findings by Pavet et al. led to the conclusion that through an increased glutathione content and higher redox levels (more glutathione reduced than oxidized), AA deficiency triggers defense responses in the SA signaling pathway, which is independent of H_2O_2. The authors, however, suggested that the defense signaling does not depend on SA.

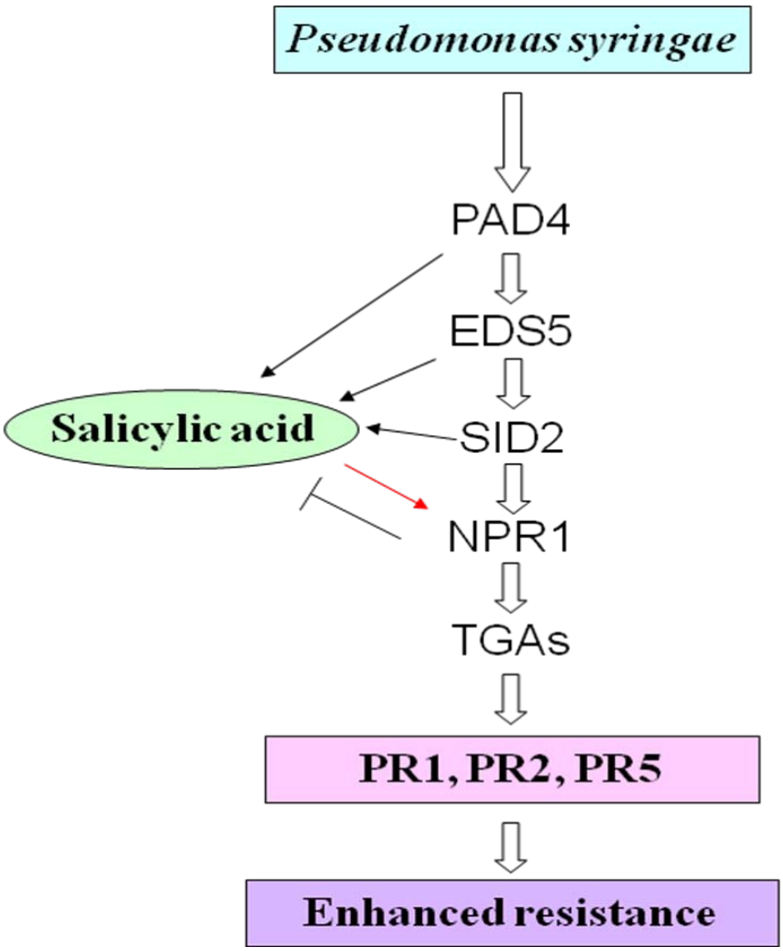

Figure 1-2: A strongly simplified version of the salicylic acid signaling pathway. Upon pathogen attack by for example *Pseudomonas syringae*, the *PAD4* and *EDS5* genes are required for the accumulation of salicylic acid, which, in response to pathogen challenge, is synthesized via SID2. Salicylic acid activates the key regulator NPR1, which in turn is necessary to activate TGA transcription factors that bind to *PR* genes to regulate their transcription. PR proteins have antimicrobial activity, resulting in a defense response.

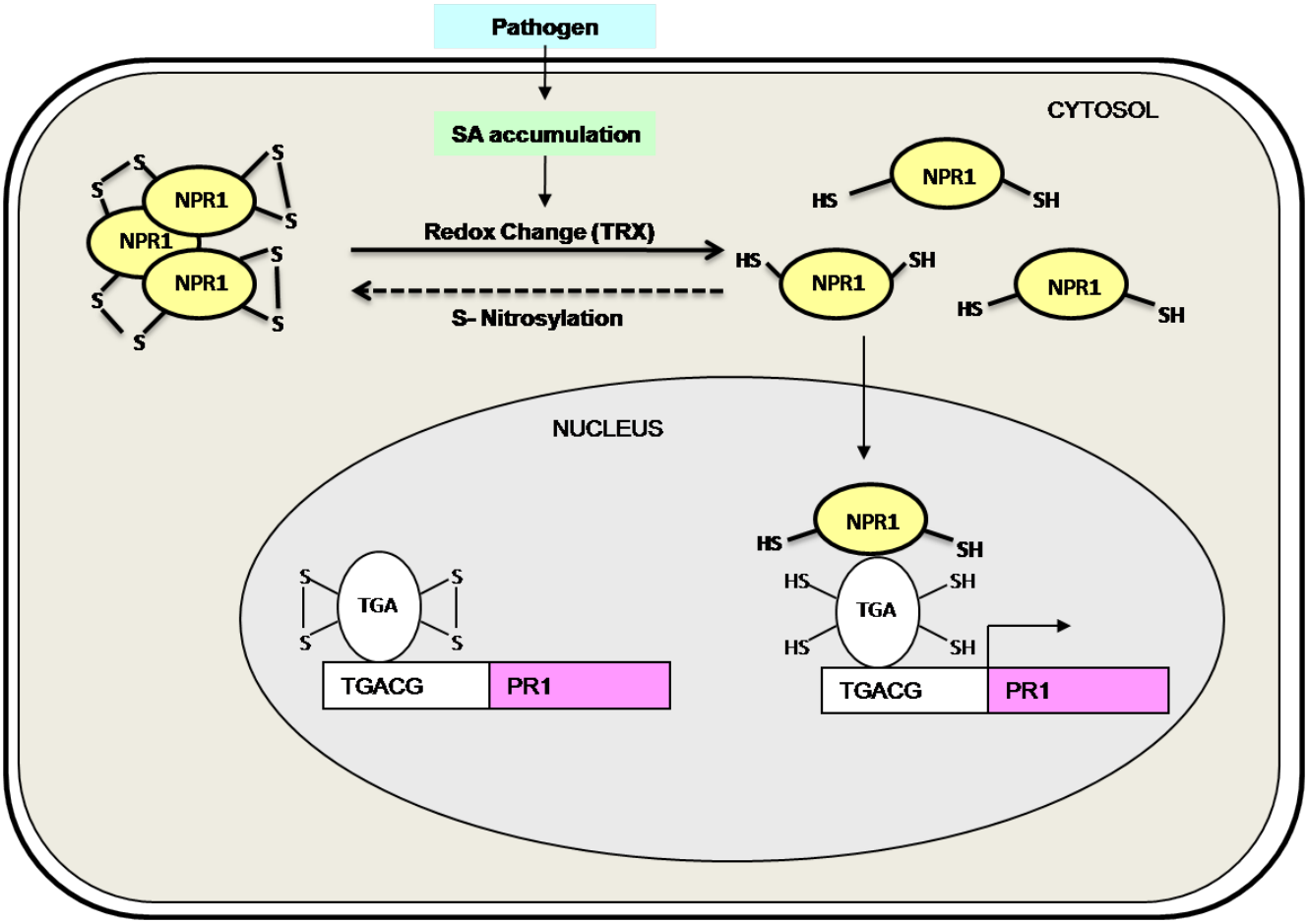

Figure 1-3: Proposed mechanism of salicylic acid regulating NPR1 function. In the absence of pathogens, NPR1 is present in form of inactive oligomers (the oxidized form of NPR1) located in the cytosol. TGA transcription factors recognize SA-responsive promoter elements (TGACG). This is, however, not sufficient to activate *PR-1* gene expression. Upon pathogen infection, SA accumulates and plant cells undergo a shift to more reducing conditions, which are necessary to reduce NPR1 from its inactive oligomeric form to its reduced active monomers through the reduction of intermolecular disulfide bonds. Thioredoxins (TRX) have been shown to play a role in this process. NPR1 monomers translocate into the nucleus, where they interact with TGAs. Binding of NPR1 to TGAs stimulates the DNA-binding activity of these transcription factors, resulting the activation of *PR-1* gene expression. Evidence suggests that the level of active NPR1 monomers is regulated through the process of S-nitrosylation, Adapted from Pieterse and Van Loon 2004 and Tada et al. 2008.

1.1.5 Specific objectives of the study

Specific Objective 1: Elucidate how ascorbic acid influences flowering time in *Arabidopsis thaliana* by investigating the effect of ascorbic acid on PHYB, and by generating double mutants between *vtc1-1* and mutants in the photoperiodic and autonomous flowering pathways.

To accomplish this objective we tested whether artificially increasing the AA content in the early-flowering *phyB-9* mutant by spraying plants with the AA precursor L-galactose would alter flowering time. Furthermore, the *vtc1-1* mutant was crossed to the late-flowering photoperiodic pathway mutants *gi-1*, *co-2,* and *ft-1* and to the autonomous pathway mutant *fca-1*, which is also delayed in flowering.

Specific Objective 2: Elucidate the pathway through which AA deficiency confers enhanced disease resistance to virulent *P. syringae* by (i) identifying the factors that stimulate SA biosynthesis when AA levels are low; (ii) identifying through which biosynthetic pathway SA is synthesized and accumulated in *vtc* mutants; and (iii) determining how expression of these traits is influenced by pathogen infection.

To achieve this objective, the SA content in the wild type and four *vtc* mutants was measured in non-infected and *P. syringae*-challenged plants. Furthermore, double mutants between *vtc1-1* and the SA biosynthesis and signaling mutants *pad4-1*, *eds5-1*, and *npr1-1*, respectively, were generated and bacterial growth and SA content in homozygous single and double mutants were evaluated. Finally, the H_2O_2 content in *vtc* mutants, the double mutants, and in wild-type and *vtc1-1* plants sprayed with a precursor of AA was determined.

1.2 MATERIALS AND METHODS

1.2.1. Plant material and growth conditions

Arabidopsis thaliana L. Heynh wild-type ecotype Columbia-0 (Col WT) and previously described *Arabidopsis* mutants *vtc1-1*, *vtc2-1*, *vtc3-1*, *vtc4-1* were kindly provided by P. Conklin (Conklin et al., 1996; Conklin et al., 2000; Conklin, 2001) were grown in a growth chamber (Percival, Perry, IA). Mutant plants were backcrossed to Col wild type four times (*vtc1-1*), three times (*vtc3-1*) or two times (*vtc2-1*, *vtc4-1*). The flowering time mutants *phyB-9* (CS6217), *gi-1* (CS3123), *co-2* (CS175, CS55), *ft-1* (CS56), and *fca-1* (CS167) and the wild type Landsberg *erecta*-0 (CS20; L*er* WT) were obtained from the Arabidopsis Biological Resource Centre (ABRC). Plants were grown on Metromix 360 soil (BFG Supplies, Burton, OH) in replicate flats containing 32 inserts with wild-type controls and mutants always present on the same flat. Temperature in the chamber was 23°C at day and at night. Plants were grown under long-day (LD, 16 h light/8 h dark, growth chamber lights turned on at 6:00 AM and turned off at 10:00 PM) and short-day conditions (SD, 10 h light/14 h dark, growth chamber lights turned on at 6:00 AM and turned off at 4:00 PM), respectively, at a light intensity of 160 μmol photons m^{-2} s^{-1} (fluorescent bulbs).

Two-week-old plants were harvested by pooling above-ground tissue of seedlings. When plants were three weeks old, either entire rosettes were harvested or rosette leaves of similar age were marked using a soft marker. Rosette leaves were then pooled from two to four individual plants. Plants were only used once for tissue harvest to avoid gene expression alterations due to wound effects. In all cases, plant tissue was collected 4 h after growth chamber lights turned on and immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

1.2.2. Generation and identification of *vtc1-1* and flowering time double mutants

The *vtc1-1* mutant (Conklin et al., 2000, Col background) was crossed with *gi-1* (Col background; Fowler et al., 1999), *co-2* (L*er* background; Putterill et al., 1995), *ft-1* (L*er* background; Kardailsky et al., 1999), and *fca-1* (L*er* background; Macknight et al., 1997), respectively. F_1 progeny of the crosses were allowed to self. F_2 progeny were

screened for AA deficiency (Conklin et al., 2000) and DNA was extracted from progeny that scored as AA-deficient. Per plant, two PCR reactions were carried out to identify homozygous *vtc1-1 gi-1*, *vtc1-1 co-2*, *vtc1-1 ft-1*, and *vtc1-1 fca-1* double mutants using the primers listed in Table 1-1. F_3 seeds from homozygous double mutants were used for experiments. The individual Col and L*er* wild types and the Col/L*er* crosses were used as controls. To minimize genetic variability, resulting from crossing two different backgrounds, seeds were pooled from 12 different crossing events of the Col and L*er* wild-type controls (Col/L*er*; Miller et al., 2007) and at least three independent double mutants (except for *vtc1-1 co-2*) were evaluated for flowering time.

Table 1. Sequences of oligonucleotide primers used for mutant identification of the flowering pathway genes.

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Name	Primer sequence	ATG number
CO-2-F	5'-CCACGTGTAGGCACTCAGGA-3'	AT5G15840
CO-2-R	5'-GAACAGCCACGAAGCAACCT-3'	
FCA1-F	5'-CCCGTTAGGTGGTTATGGTGTTCC-3'	AT4G16280
FCA1-R	5'-TTGGTTTGGTTGCTGCATAGACTG-3'	
FT sequ-F	5'-CCTGCTACAACTGGAACAACC-3'	AT1G65480
FT sequ-R	5'-ATAGGCATCATCACCGTTCG-3'	
GI sequ-F*	5'-CTGAACCCTTGGAAGCCTACCT-3'	AT1G22770
GI sequ-R*	5'-ATGCACTTGCGAGAATCACCAG-3'	
GI sequ-F2*	5'-GCGCTATGCAATGTCGTATCTG-3'	
VTC1-F2	5'-ACATTTTTAGCAGCTGGTATTGAG-3'	AT2G39770
VTC1-R2	5'-AGGTAAGAACTGGCAGACTAAAG-3'	

*PCR product was generated using GI sequ-F and GI sequ-R primers, but GI sequ-F2 was used for sequencing.

1.2.3 Generation and identification of double mutants of *vtc1-1* and mutants defective in SA biosynthetic or signaling genes

The *vtc1-1* mutant was crossed with *pad4-1*, *eds5-1*, *npr1-1*, and *fca-1*, respectively, as described above. Per plant, two PCR reactions were carried out to identify homozygous double mutants using the primers listed in Table 2. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and

sequenced at Macrogen, Inc. (Korea). F_3 and F_4 plants of homozygous double mutants were used for experimental analyses.

Table 2. Sequences of oligonucleotide primers used for mutant identification of the SA biosynthesis/signaling genes.

Name	Primer sequence	ATG number
EDS5-F	5'-ACGTTGCTTAAGTCCCTGATGATT-3'	AT4G39030
EDS5-R	5'-ACGTCCCGCCTGGTGAAC-3'	
FCA1-F	5'-CCCGTTAGGTGGTTATGGTGTTCC-3'	AT4G16280
FCA1-R	5'-TTGGTTTGGTTGCTGCATAGACTG-3'	
NPR1-F	5'-GAGGACACATTGGTTATACTC-3'	AT1G64280
NPR1-R	5'-CAAGATCGAGCAGCGTCATCTTC-3'	
PAD4-F2	5'-ATTGTTCGGGCTCCTATTCTG-3'	AT3G52430
PAD4-R2	5'-TGCTCGCGTATCTGCTTCTCAC-3'	
VTC1-F2	5'-ACATTTTTAGCAGCTGGTATTGAG-3'	AT2G39770
VTC1-R2	5'-AGGTAAGAACTGGCAGACTAAAG-3'	

1.2.4. Bacterial infection with virulent *Pseudomonas syringae* pv. *maculicola* ES4326

The virulent strain of *P. syringae* pv. *maculicola* ES4326 was kindly provided by Dr. Gregory Martin (Boyce Thompson Institute of Plant Research, Ithaca, NY). Inoculation of plants was performed using the syringe infiltration (Barth et al., 2004) or the dipping method (Katagiri et al., 2002) using an inoculation titer of 10^5 Colony Forming Units (CFU) per ml and 10^7 CFU per ml, respectively. In case of the dipping method, the plants were grown in a 3-inch square pot with a mesh. This helped contain the soil during inversion of the pot while dip inoculating. Plants were grown on a small mound of soil (about 0.5-1 inch) held by a fine nylon mesh placed on top that was fixed by a rubber band. The seeds were then sown on the soil covered with mesh. After germination (for one week flats were covered with domes), the domes were removed and excess germinated seedlings were thinned out so that each pot contained only one seedling. Once the plants were three weeks old, they were used for bacterial inoculation. Each pot was turned upside down and dipped into a solution containing the inoculum along with 0.04% Silwet L-77 (Lehle Seed, Round Rock, TX), a surfactant. Plants were dipped for approximately 30 sec until all the leaves were equally and thoroughly wetted

with the bacterial suspension. Mock controls were either infiltrated with 10 mM $MgCl_2$ or dipped in water, containing 0.04% of Silwet L-77.

For syringe infiltration, three-week-old plants were infiltrated with *P. syringae* that were grown for 2 days at 28°C in LB agar plates supplemented with 100 µg/ml streptomycin. Fresh single colonies were picked from the plates and grown in 3 ml liquid LB with 100 µg/ml streptomycin for 16 hours at 28°C with shaking at 250 rpm. Bacterial cells were collected by centrifugation at 5000 rpm for 15 mins. The bacterial pellet washed twice with 10 mM $MgCl_2$ or distilled water. Finally the cell suspension was brought to a desired OD of either 10^5 or 10^7 CFU ml^{-1} respectively. Approximately 20 µl of the bacterial suspension were inoculated to the underside of the leaves with a 1 ml plastic syringe without a needle.

Mock and bacterial inoculated leaf discs were collected at 0, 24, 48, 72, 96 h after inoculation for bacterial count. The remaining tissue was frozen in liquid nitrogen for RNA isolation. For bacterial count, leaf discs of 0.5 cm in diameter were punched using a cork borer. Four leaf discs per replicate per genotype were collected in an Eppendorf tube and macerated using plastic pestles in 0.3 ml of 10 mM $MgCl_2$ or distilled water. Serial dilutions of the samples collected at the respective time points were plated on LB agar plates containing streptomycin, colonies were counted and CFU cm^{-2} leaf areas were calculated.

1.2.5. Exogenous application of L-Galactose

Wild-type, *vtc1-1*, and *phyB-9* mutant plants were grown as described above. Seven days after germination, plants were sprayed every day with either water or 10 mM L-galactose dissolved in water. Ten milliliter of the galactose solution were sprayed onto one flat containing 32 plants, while each plant received two sprays (approximately 150 µl). When plants were three weeks old, rosette leaves were harvested for determination of AA, H_2O_2 and SA content.

1.2.6. Ascorbic acid content assay

The amount of total and reduced AA was determined in whole rosettes of three-week-old plants (fresh weight was approximately 100-200 mg) following the iron reduction assay (Kampfenkel et al., 1995; Dowdle et al., 2007). This assay is based on the reduction of Fe^{3+} to Fe^{2+} by AA and the spectrophotometric detection of Fe^{2+} complexed with 2,2'-dipyridyl. Dehydroascorbate is reduced to AA by pre-incubation with dithiothreitol (DTT). Excess DTT was removed by N-ethylmaleimide (NEM) and total AA was determined. The amount of reduced AA was determined by replacing DTT and NEM with water.

Leaf tissue was completely crushed in liquid nitrogen in a paint shaker with three metal ball bearings in an 1.5 ml Eppendorf tube. To extract AA, 1 ml of 0.1M HCl containing 1 mM EDTA was added to the samples which were centrifuged for 15 min at 15,600 g at 4˚C. The supernatant was transferred to a new tube and centrifuged again to pellet any excess leaf material. The samples were then placed on ice. The assay was carried out in a 96-well plate with 10 µl of sample assayed in each well. Reduced and total AA were assayed simultaneously in separate plates. 10 microliter of 10 mM DTT were added to each sample to assay for total AA along with 20 µl of 0.2 M sodium phosphate buffer (pH 7.4). The samples were mixed and incubated for 15 min at 42˚C. To assay for reduced AA, samples received 30 µl of the phosphate buffer along with 10 µl of distilled water. After incubation of the total AA samples, the reaction was stopped by adding 10 µl of 0.5% NEM, to bind excess DTT, followed by mixing and incubating for 1 min at room temperature. To both plates, 50 µl of 10% TCA, 40 µl of 42% H_3PO_4, and 40 µl of 4% 2, 2' dipyridyl (dissolved in 70% ethanol) were added and mixed vigorously. Note that after addition of each component samples were mixed by pipetting. To control samples not containing dipyridyl, an equal amount of distilled water was added. Finally, 20 µl of 3% $FeCl_3$ were added to all samples followed by mixing through pipetting. The samples were then incubated at 42˚C in an incubator for 40 min and the absorbance was measured at 525 nm in a plate reader.

To account for absorbance caused by the presence of anthocyanins, a “dipyridyl control” not containing dipyridyl was included for samples and standards. Absorbance values of the samples were corrected by subtracting the absorbance value of the blank

(containing HCl instead of extract) and the dipyridyl controls. Absorbencies were then plotted against a standard curve (both for reduced and total AA) of known concentrations (0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12 µmoles) to determine the AA amount of the sample extracts. The AA content was normalized to gram fresh weight of tissue and expressed as µmoles gFW^{-1}.

1.2.7. Salicylic acid content assay

Total and free SA were measured from whole rosettes (200-300 mg fresh weight) essentially as described previously (Bowling et al., 1994). Rosettes were collected in 15 ml Falcon tubes containing 4 metal ball bearings, in liquid nitrogen and the frozen tissue was crushed using a paint shaker. Briefly, after extracting the tissue in 2.5 ml of 90% methanol, 50 µl of 0.1 mM O-anisic acid (Sigma, St. Louis, MO) were added as an internal standard. Samples were shaken for 20 min at room temperature and centrifuged at 2800 g for 15 min. The supernatant was transferred to a glass tube and the previous steps were repeated after resuspending the pellet in 100% methanol. The supernatants were combined, mixed, split into two fractions and dried under vacuum in a glass desiccator for two days. To purify SA, 400 µl of 100 mM sodium acetate at pH 5.5 were added to samples for measuring free SA and 40 U of β-glucosidase suspended in sodium acetate was added to samples for measuring total SA. Samples were incubated at 37ºC for 1.5 h. An equal volume of 50% trichloroacetic acid were added to the samples followed by centrifugation at 2800 g for 15 min at room temperature. The supernatants were then partitioned twice with 1.25 ml of a mixture (100:99:1) of ethylacetate: cyclopentane:2-propanol. The organic phase was collected and dried under vacuum for two to three days. The dried samples were stored at -20ºC (no more than one week) until samples were analyzed by HPLC. The pellet was suspended in 55% methanol and the samples were passed through 0.2 µm nylon spin columns (Fisher, Pittsburgh, PA) by centrifugation at 14,000g for 2 min. Samples were then analyzed on a HP1100 HPLC System (Agilent, Santa Clara, CA) equipped with a 4.6 x 150 mm Zorbax Eclipse Column and fluorescence detector (excitation at 301 nm and emission at 412 nm and 365 nm for SA and O-anisic acid, respectively). Solvent flow was 1 ml/min. Mobile phases were A,

100% methanol, and B, 0.5% acetic acid. The solvent program consisted of 0-5 min at 30% A, 70% B, followed by a linear increase to 40% A, 60% B at 7.5 min, and 60% A, 40% at 15 min, and returning to 30% A, 70% B at 18 min.

1.2.8. Hydrogen peroxide content assay

Hydrogen peroxide production in whole rosettes of the wild type and mutants was measured as previously described (Shin and Schachtman, 2004), using the Amplex red hydrogen peroxide/peroxidase assay kit (Invitrogen, Carlsbad, CA). The stock solutions were prepared as described in the kit protocol. About 50-300 mg of tissue were harvested in 1.5 ml Eppendorf tubes and frozen immediately in liquid nitrogen. Samples were analyzed immediately after harvest. Two hundred microliters of 20 mM phosphate buffer (pH 6.5-7.0) were added to each sample. Samples were vortexed for 30 seconds and centrifuged at 10,000 rpm for 10 min at 4°C. Fifty microliters of the supernatant were used for the assay. A 100 µM working solution of Amplex Red reagent and 0.2 U/ml horseradish peroxidase (HRP) were prepared. The reagent mixture contained 0.5 µl of 10 mM Amplex Red reagent stock solution, 1.0 µl of 10 U/ml HRP stock solution and 4.85 ml of 1X reaction buffer for 1 reaction. Fifty microliters of this working solution were added to 50 µl of sample, standards (5 µM, 2 µM, 1 µM, 0.5 µM, 0.1 µM and 0.05 µM) and controls (containing 50 µl of extraction buffer instead of the extracts) in a 96-well plate. Samples were incubated in the dark for 30 mins and the absorbance was measured at 590 nm using a plate reader.

1.2.9. Extraction of RNA, cDNA synthesis and gene expression analysis

Isolation of total RNA, cDNA synthesis and transcript analyses using semi-quantitative PCR and quantification of PCR products of defense-related genes were essentially performed according to Kotchoni et al (2009).

1.2.9.1 Extraction of RNA

Leaf samples were flash frozen in liquid nitrogen and stored in 1.5 ml Eppendorf tubes until further analysis. For RNA extraction, samples were first crushed in liquid

nitrogen followed by the addition of 1 ml TRI Reagent (Molecular Research Centre, Inc) to each tube and the samples were stored at room temperature for 5 min. Then, 0.2 ml of chloroform were added to each tube followed by vigorous mixing using a vortex and storing at room temperature for 15 min. Samples were then centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase was transferred to a clean tube and 0.5 ml of isopropanol were added to the supernatant, vortexed and stored at room temperature for 10 min. Samples were centrifuged at 12,000 g for 8 min at 4°C. The obtained RNA pellet was carefully washed with 1 ml 75% ethanol, centrifuged for 5 min at 12,000 g at 4°C. The RNA pellet was air dried for 5-10 min and then solubilized by adding 30 µl of sterile water and incubation at 55-60°C for 10 min. RNA concentrations were determined using a Nanodrop Spectrophotometer, and stored in -80°C until further analysis.

1.2.9.2 cDNA synthesis

For cDNA synthesis, 5 µg of RNA were used in a 20 µl reaction. Two microliters of 20 µM Oligo dT primer and 1 µl of 10 mM dNTP were added and incubated at 65˚C for 5 min. The reaction was stopped by placing the tubes on ice. To each tube, 4 µl of reaction buffer and 2 µl of 0.1 DTT were added. The samples were incubated for 2 min at 38˚C in a water bath. To each reaction, 1 µl of Superscript II RT (Reverse Transcriptase, 200 units/µl) were added and incubated for an hour at 38˚C for cDNA synthesis. The reaction was stopped by heating the tubes at 70˚C for 15 min. The cDNA was stored at -80˚C until further analysis.

1.2.9.3 Transcript analyses

cDNAs synthesized from specific experiments were analyzed by using gene-specific primers listed in Table 1-3. A cDNA fragment generated from *ACTIN* or *TUBULIN* served as an internal control.

Table 3. Sequences of oligonucleotide primers used for gene expression analysis.

Name	Primer sequence	ATG number
ACTIN2-F	5'-ATGGCTGAGGCTGATGATATTCAAC-3'	AT3G18780
ACTIN2-R	5'-GAAACATTTTCTGTGAACGATTCCT-3'	
EDR1-RT-F1	5'-TGCGGATTACATGTCTTCTGAGGA-3'	AT1G08720
EDR1-RT-R1	5'-CTGCTTTGCGGAGTCTGTGGAT-3'	
EDS1-RT-F1	5'-TTCCTCGTATGGCTTTCCTTTGA-3'	AT3G48090
EDS1-RT-R1	5'-AGTTAGCCGGTGTTTTTGACGAG-3'	
EDS5-RT-F1	5'-CGTGGCCGTTTATCCTTGTTG-3'	AT4G39030
EDS5-RT-R1	5'-AGCGAGTGCAGAGATCTTCCATAG-3'	
MKK2-F	5'-TGATCAGCTGAGCTTGTCGG-3'	AT4G29810
MKK2-R	5'-ATGGTGATATTATGTCTCCC-3'	
MPK3-F	5'-ATACGGAGACGAACGAGCTAG-3'	AT3G45640
MPK3-R	5'-CGCATTTGATGAACATGGTCT-3'	
MPK4-F	5'-ATGTCGGCGGAGAGTTGTTTCG-3'	AT4G01370
MPK4-R	5'-TCACACTGAGTCTTGAGGATTG-3'	
MPK6-F	5'-ATGGACGGTGGTTCAGGTCAA-3'	AT2G43790
MPK6-R	5'-TTGAAAGCAAGCGCCTCGCGG-3'	
NPR1-F	5'-AGCAGAGTGAGACCGCCGCCTAAA-3'	AT1G64280
NPR1-R	5'-CTCCTTCCGCATCGCAGCAACA-3'	
PAD4-F2	5'-ATTGTTCGGGCTCCTATTCTG-3'	AT3G52430
PAD4-R2	5'-TGCTCGCGTATCTGCTTCTCAC-3'	
PAL1-RT-R1	5'-CGAAAACCGCCGACAAAATCTC-3'	AT2G37040
PAL1-RT-F1	5'-TGCCACACTCCGCCACAAG-3'	
PR1-F	5'-CCCGCTCAACCGCCAAAAG-3'	AT2G19990
PR1-R	5'-CAAATCACCCAAATATACTCATCA-3'	
PR2-F	5'-GAGCTTAGCCTCACCACCAA-3'	AT3G57260
PR2-R	5'-TGTACCGGAATCTGACACCA-3'	
PR5-F	5'-GATTCCGCCACCGTATTCACTTTA-3'	AT1G75030
PR5-R	5'-GCTCTTACACGCCGCGACACTTC-3'	
SID2-RT-F1	5'-GCTGCTTCTAGTGCTCGTGATA-3'	AT1G74710
SID2-RT-R1	5'-GGTCCCGCATACATTCCTCTA-3'	
TUB2-RT-F2	5'-CTCAAGAGGTTCTCAGCAGTA-3'	AT5G62690
TUB2-RT-R2	5'-TCACCTTCTTCATCCGCAGTT-3'	

1.2.10. Statistical Analyses

Data were expressed as mean values ± SE. Experiments were repeated at least three times. *P* values were determined by Student's *t*-test analysis.

1.3. RESULTS

1.3.1 The role of ascorbic acid in the control of flowering time

1.3.1.1 Ascorbic acid deficiency does not alter the expression of the flowering repressor PHYB

Kotchoni and coworkers reported that *vtc* mutants grown under both LD and SD had higher mRNA levels of *PHYA, CRY1, CRY2,* which are positive regulators of flowering, and low mRNA levels of *PHYB*, which represses flowering (Cerdan and Chory, 2003; El-Din El-Assal et al., 2003; Liu et al., 2008; Kotchoni et al., 2009). To clarify whether AA deficiency directly alters the expression of *PHYB* thus causing the *vtc* mutants to flower early, a possible effect of AA on *PHYB* using the early flowering *phyB-9* mutant (Col background) was investigated. To approach this, the AA content in these mutants was artificially increased by spraying plants with the AA precursor L-galactose (L-Gal) to test if the altered AA content would change flowering time. Although leaf AA levels were significantly elevated in L-Gal-sprayed compared to water-treated *phyB-9* plants (Fig. 1-4A), flowering was not affected (Fig. 1-4 B and C), suggesting that functional *PHYB* is required for delayed flowering through degradation of *CO*, as expected (Cerdan and Chory, 2003; El-Din El-Assal et al., 2003). However, this experiment also suggests that early flowering in *phyB* mutants is AA-independent. This is supported by the fact that *phyB* mutants are not AA-deficient. However, note that the AA content decreases more rapidly in *phyB* mutants than in the wild type with increasing age, which correlates with the early flowering and senescence phenotype in these mutants (Fig.1-4D).

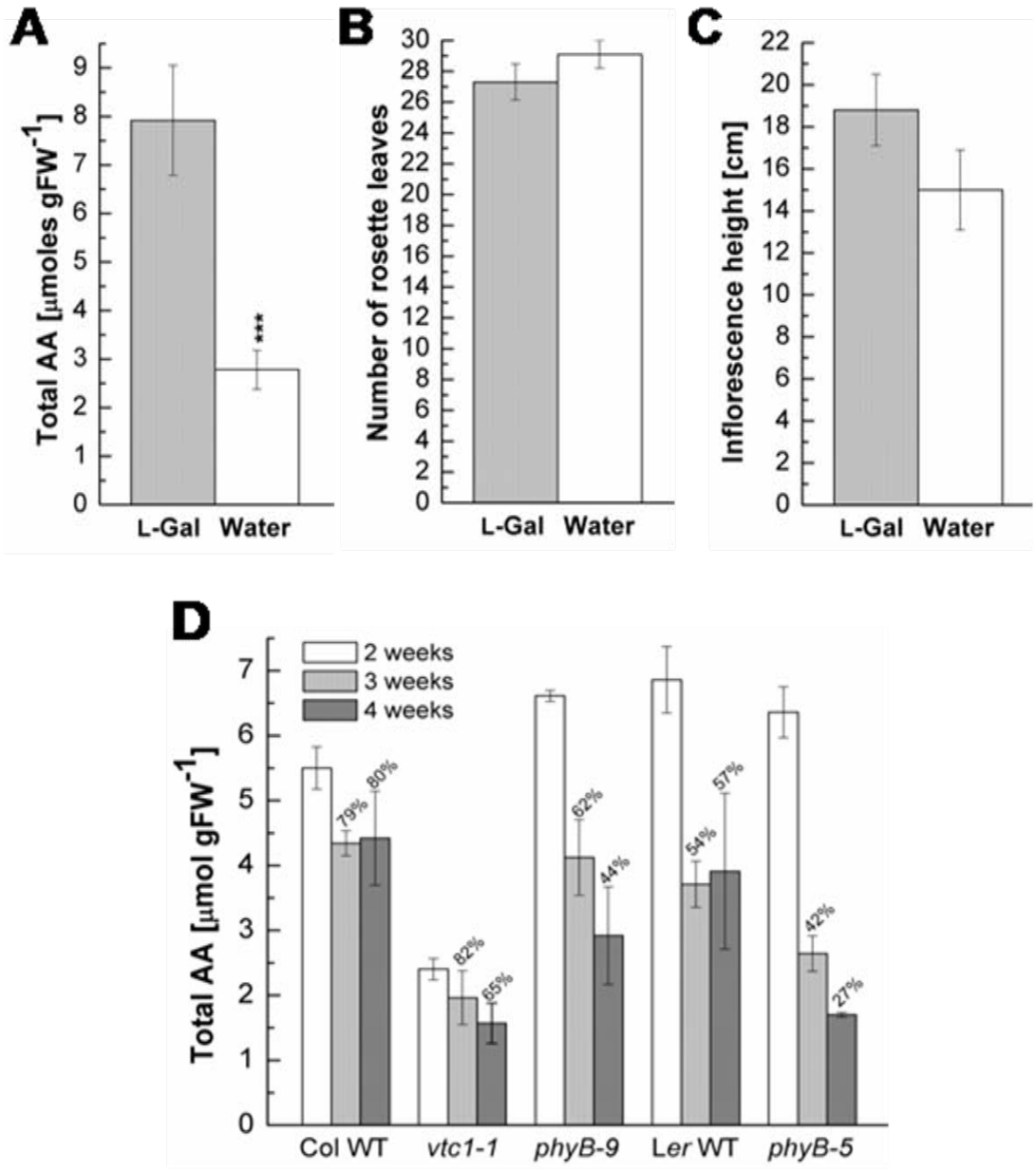

Figure 1-4: Effect of L-galactose (L-gal) treatment on flowering time in *phyB-9* mutants (A-C) and developmental changes in the ascorbic acid (AA) content in wild type, *vtc1-1* and *phyB* mutants grown under long days.
(A) Total ascorbic acid (AA) content in four-week-old *phyB-9* plants sprayed with L-Gal or water. Means ± SE of three independent replicates are shown. ***$P < 0.001$, Student's *t*-test. (B) Number of rosette leaves of four-week-old L-Gal- and water-treated *phyB-9* mutants. Data represent means ± SE of 16 independent replicates. (C) Inflorescence height in four-week-old L-Gal- and water-sprayed *phyB-9* mutants. Results illustrate means ± SE of 16 independent replicates. (D) Total ascorbic acid content in Columbia-0 wild type (Col WT), *vtc1-1* (Col background), *phyB-9* (Col background), Landsberg *erecta*-0 wild type (L*er* WT), and *phyB-5* (L*er* background) during development. Two, three, four and five weeks after sowing, whole rosettes were harvested 4 h after growth chamber lights turned on. Percentages on top of bars indicate differences compared to the respective two-week-AA content. Means ± SE of three independent replicates are shown. (After Kotchoni et al., 2009)

1.3.1.2 *vtc1-1 gi-1, vtc1-1 co-2, vtc1-1 ft-1* and *vtc1-1 fca-1* double mutants exhibit delayed flowering despite their ascorbic acid deficiency

Work by Kotchoni et al. (2009) established that the early flowering phenotype in the *vtc* mutants was associated with the two-fold higher transcript levels of the photoperiodic pathway genes *GI, CO,* and *FT* both under SD and LD. In contrast, mRNA levels of the autonomous pathway gene *FCA* did not differ or were only slightly different in the *vtc* mutants compared to the wild type grown under SD or LD. However, Kotchoni et al. found that the *FLC* mRNA levels were 30% lower in the SD-grown *vtc1-1, vtc2-1* and *vtc3-1* mutants and more than two-fold lower in all *vtc* mutants grown under LD compared to the wild type. These expression patterns correlated with transcript levels of the floral meristem identity gene *LFY* with the exception of *vtc2-1* (Kotchoni et al., 2009).

To address whether there is a genetic interaction between the AA biosynthetic gene *VTC1* and genes in the photoperiodic and autonomous pathways, *vtc1-1* was crossed to the late flowering photoperiodic pathway mutants *gi-1*, *co-2*, and *ft-1*, and the autonomous pathway mutant *fca-1*, which is also delayed in flowering (Koornneef et al., 1991; Putterill et al., 1995; Macknight et al., 1997; Fowler et al., 1999; Suárez-López et al., 2001). If AA specifically acts in the autonomous pathway, *vtc1-1 gi-1*, *vtc1-1 co-2*, and *vtc1-1 ft-1* double mutants should exhibit a slight promotion of flowering compared to *gi-1*, *co-2* and *ft-1* single mutants due to the activity of the autonomous pathway in *vtc1-1*. Likewise, *vtc1-1 fca-1* double mutants are expected to exhibit earlier flowering than *fca-1* single mutants due to the enhanced activity of the photoperiodic pathway measured in *vtc1-1* (Kotchoni et al., 2009). Although *FCA* transcript levels were largely unaltered in the *vtc* mutants compared to the wild type (Kotchoni et al., 2009), *vtc1-1 fca-1* double mutants were nevertheless generated because the transcript analysis by Kotchoni et al. did not differentiate between the *FCA* splice variants (Macknight et al., 2002).

All double mutants displayed a delayed flowering phenotype despite their AA deficiency (Fig. 1-5A). Note that *vtc1-1 fca-1* had approximately 70% of the Col/L*er* wild-type AA content, suggesting that *fca-1* can partially suppress the *vtc1-1* mutation. All homozygous double mutants had a similar number of rosette leaves as the *gi-1, co-2*,

ft-1 and *fca-1* single mutants, respectively, whereas *vtc1-1* developed significantly fewer rosette leaves than the Col wild type under LD and SD (Fig. 1-5B and C). Note that the SE bars for both the AA and flowering data in the Col/L*er* wild type and in the double mutants of two different backgrounds are comparable to the SE bars of the respective wild-type controls, suggesting low genetic variability in the pools of plants used for the experiments.

In summary, the genetic analysis demonstrates that the *gi-1*, *co-2*, *ft-1*, and *fca-1* mutations are all epistatic to *vtc1-1*, suggesting that *GI*, *CO*, *FT*, and *FCA* play a role in the promotion of flowering of *vtc1-1*. However, AA does not appear to have a specific role in any of the known flowering pathways.

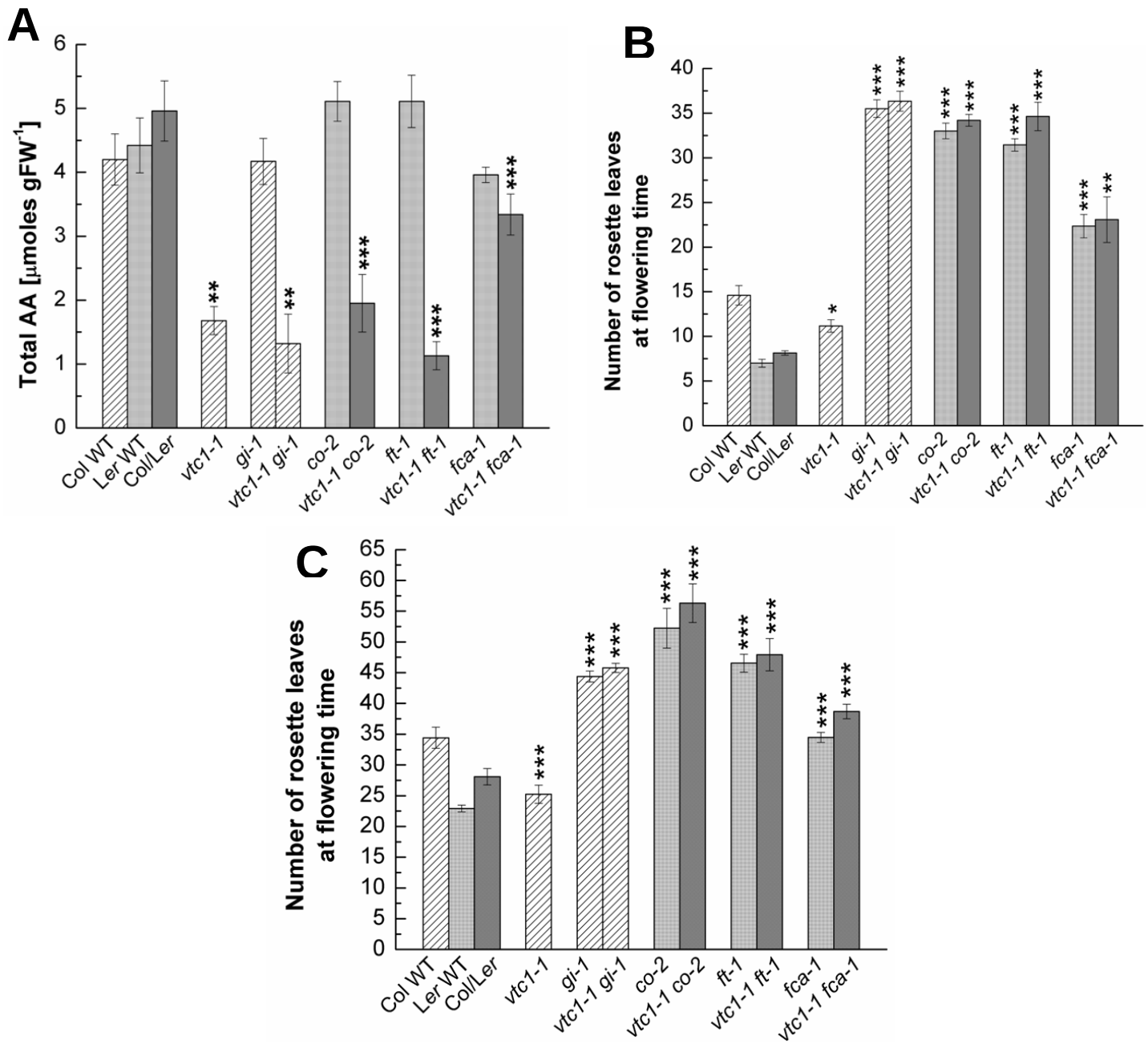

Figure 1-5: Effect of the *vtc1-1* mutation on flowering time and ascorbic acid content in the background of photoperiodic and autonomous pathway mutants.
Plants were grown under long days and short days. (A) Total ascorbic acid (AA) content in three-week-old wild-type controls, single and double mutants grown under long days. Means ± SE of three to six independent replicates per genotype are shown. (B) Total rosette leaf number of all genotypes at flowering time under long days. (C) Total rosette leaf number of all genotypes at flowering time under short days. Results depict means ± SE of nine to seventeen independent plants per genotype. Shading patterns indicate plants of the same genetic background, allowing for easier statistical comparison of single and double mutants to their respective wild-type controls. Asterisks denote significant differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. (After Kotchoni et al., 2009)

1.3.2 The role of ascorbic acid in pathogen defense against *Pseudomonas syringae*

1.3.2.1. *vtc* mutants have constitutively high mRNA levels of *NPR1* and *PR* genes that correlate with increased SA levels and enhanced resistance to *P. syringae*

Rosette leaves of the AA-deficient mutants *vtc1-1* and *vtc2-1* contain approximately 30% and 25% of the wild-type AA content, respectively, whereas the *vtc3-1* and *vtc4-1* mutants have 40% and 50%, respectively (Fig. 1-6A). As reported previously (Kotchoni et al., 2009), the diminished AA content leads to moderate oxidative stress, which is indicated by the increase in H_2O_2 content. The H_2O_2 content in *vtc1-1*, *vtc2-1*, and *vtc3-1* mutants was about 60% to 70% higher than in the wild type. In contrast, H_2O_2 levels were the same in the wild type and *vtc4-1* (Fig. 1-6B).

The *vtc1-1* and *vtc2-1* mutants were reported to have constitutively higher transcript and protein levels of defense genes and increased resistance to virulent pathogens (Barth et al., 2004; Pavet et al., 2005). To test whether this is a general phenomenon of AA deficiency, we measured transcript levels of *NPR1*, *PR1*, *PR2*, and *PR5* in the wild type and *vtc* mutants. *NPR1* mRNA levels were two to three times higher in all *vtc* mutants than in the wild type. Transcript levels of *PR1* were similarly elevated in the *vtc* mutants except for *vtc4-1*. Compared to the wild type, the *vtc1-1* and *vtc2-1* mutants contained two-fold higher transcript levels of *PR2*, whereas *PR2* mRNA levels were unchanged in *vtc3-1* and *vtc4-1*. Finally, *PR5* mRNA levels were more than three times higher in all *vtc* mutants than in the wild type (Fig. 1-6C).

PR gene expression can be activated through salicylic acid (SA)-dependent signaling (Shah, 2003; Durrant and Dong, 2004; Glazebrook, 2005). We therefore asked whether the higher transcript levels of defense genes correlate with elevated levels of SA and enhanced resistance to virulent *P. syringae*. The *vtc* mutants did not differ in their free SA content. However, the *vtc1-1*, *vtc2-1*, and *vtc3-1* mutants contained about four times and 2.5-times more SA glucoside, respectively, than the wild type. In contrast, *vtc4-1* had wild-type SA levels (Fig. 1-6D). The elevated SA content in the mutants correlated with enhanced resistance to *P. syringae* infection in the *vtc* mutants. The *vtc1-1* and *vtc2-1* mutants significantly diminished growth of *P. syringae* already 24 h post inoculation, followed by *vtc3-1* that exhibited increased resistance 48 hours and *vtc4-1* supporting less growth 72 hours post inoculation (Fig. 1-6E). Note that *vtc4-1* does

not contain constitutively elevated SA as the other *vtc* mutants. However, it does diminish growth of *P. syringae* at later points in the infection process.

Our data suggest a correlation between the degree of AA deficiency, H_2O_2 and SA accumulation, mRNA levels of defense genes and resistance to *P. syringae*. The greater the extent of AA deficiency, the more pronounced are SA-dependent defense signaling and resistance. Since AA levels of 40% or less of the wild type result in a substantial constitutive increase in SA, we asked through which biosynthetic pathway SA is accumulated in the *vtc* mutants.

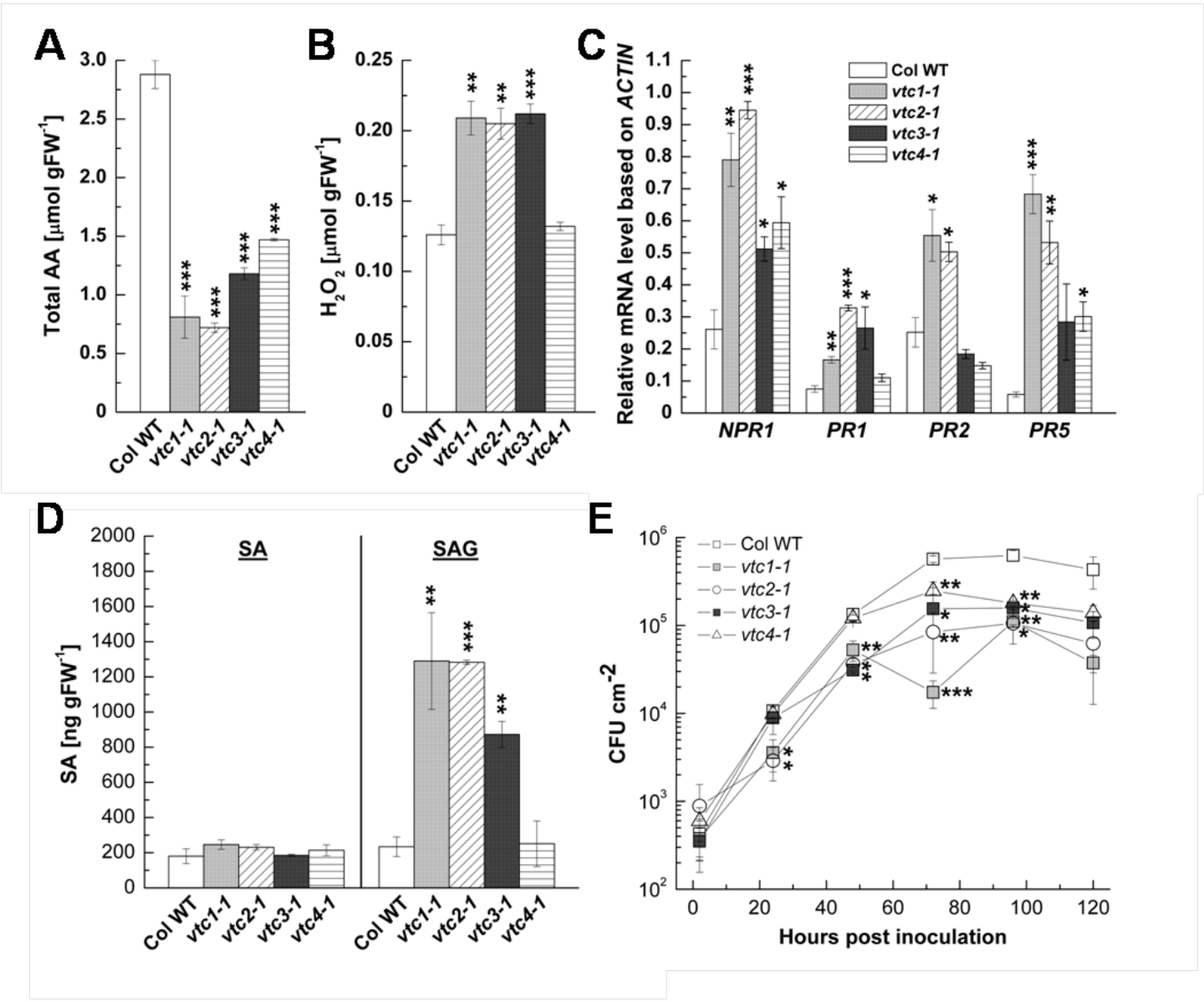

Figure 1-6: Physiological characterization of three-week-old ascorbic acid-deficient *vtc* mutants.

(A) Total ascorbic acid (AA) and (B) hydrogen peroxide (H_2O_2) content based on gram fresh weight (FW) in whole rosettes of Columbia-0 wild-type (Col WT) and *vtc* mutant plants. (C) Transcript levels of *NPR1, PR1*, *PR2*, and *PR5* normalized to *ACTIN*. (D) Free salicylic acid (SA) and SA-glucoside (SAG, the conjugated form) in whole rosettes of wild-type and *vtc* mutant plants. (E) Bacterial growth of virulent *P. syringae* (expressed in colony forming units, CFU, per square centimeter leaf area) in the wild type and *vtc* mutants. Three-week-old plants were syringe-infiltrated with a bacterial suspension of 10^5 CFU ml^{-1} and leaf discs were collected at the indicated time points. Results depict means ± SE of three to five independent plants per genotype. Asterisks indicate significant differences between the wild type and mutants. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. (After Mukherjee et al., 2010)

1.3.2.2. Double mutants of *vtc1-1* and mutants defective in SA biosynthetic or signaling genes have decreased SA levels and are more susceptible to *P. syringae*

For pathogen defense in *Arabidopsis*, SA is predominantly synthesized via isochorismate and not phenylalanine (Wildermuth et al., 2001). To test whether SA is accumulated in *vtc1-1* through the PAD4-EDS5-SID2-NPR1 biosynthesis and signaling pathway, we generated homozygous double mutants between *vtc1-1*, *pad4-1*, *eds5-1*, and *npr1-1*, respectively. All homozygous double mutants had AA levels similar to those in *vtc1-1* (Fig. 1-7A). Therefore, we expected that the double mutants also contain higher levels of H_2O_2 that are comparable to *vtc1-1*. As is illustrated in Fig. 1-7B, this is true for all three double mutants, while the single mutants have H_2O_2 levels similar to the wild type. Note, however, that the H_2O_2 content is somewhat lower in the double mutants than in *vtc1-1* single mutants.

If SA is constitutively synthesized via PAD4 and EDS5 in *vtc1-1*, we predicted that *vtc1-1 pad4-1* and *vtc1-1 eds5-1* double mutants contain low SA levels similar to the SA-biosynthesis *pad4-1* and *eds5-1* single mutants. In contrast, *vtc1-1 npr1-1* double mutants were predicted to have heightened SA levels similar to *vtc1-1*. Furthermore, all three double mutants were expected to be more susceptible to *P. syringae* infection due to their inability to either accumulate SA (*vtc1-1 pad4-1* and *vtc1-1 eds5-1*) or to mediate *PR* gene induction (*vtc1-1 npr1-1*). As expected, *vtc1-1 pad4-1* and *vtc1-1 eds5-1* double mutants were SA-deficient, containing free SA (Fig. 1-7C) and SAG (Fig. 1-7D) levels similar to the *pad4-1* and *eds5-1* single mutants. Unexpectedly, the *vtc1-1 npr1-1* double mutant had an SAG content that was approximately twice has high as that of the wild type and *npr1-1* single mutants (Fig. 1-7D), whereas the double mutant did not differ from the wild type in respect to the amount of free SA (Fig. 1-7C). However, the SAG content, although significantly higher than in the wild type, was more than two times lower in *vtc1-1 npr1-1* mutants compared to *vtc1-1* single mutants (Fig. 1-7D).

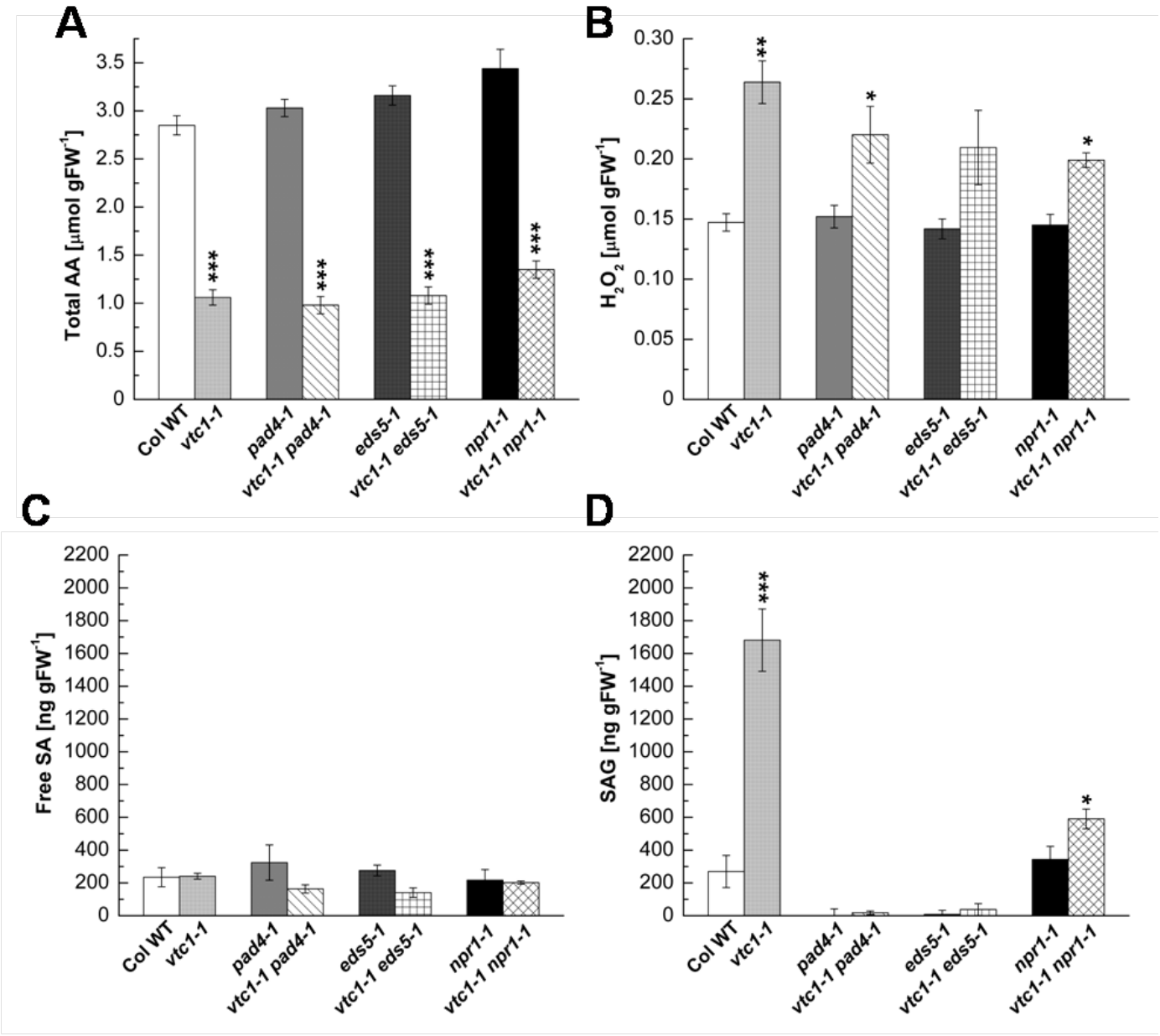

Figure 1-7: Ascorbic acid and hydrogen peroxide content in the wild type and double mutants with defects in ascorbic acid and salicylic acid biosynthesis or signaling.
(A) Total ascorbic acid (AA), (B) hydrogen peroxide (H_2O_2) content, (C) free salicylic acid (SA), and (D) SA-glucoside (SAG) content based on gram fresh weight (FW) in whole rosettes of three-week-old Columbia-0 wild-type (Col WT), *vtc1-1*, *pad4-1*, *eds5-1*, and *npr1-1* single mutants and *vtc1-1 pad4-1*, *vtc1-1 eds5-1* and *vtc1-1 npr1-1* double mutants under normal growth conditions. Results depict means ± SE of three to six independent plants per genotype. Asterisks indicate significant differences between the wild type and mutants. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. (After Mukherjee et al., 2010)

As hypothesized, all three double mutants supported more growth of *P. syringae* than the wild type and *vtc1-1*, the latter being the most resistant. The double mutants were similarly susceptible to *P. syringae* infection as the single mutants (Fig. 1-8A). We also investigated the accumulation of H_2O_2 in the various genotypes during the infection process in order to test the effect of the AA-deficient *vtc1-1* background in the double mutants. Knowledge about the H_2O_2 content is also important, because SA is known to not only potentiate H_2O_2 accumulation (Shirasu et al., 1997), but also to act downstream of H_2O_2 (Leon et al., 1995; Neuenschwander et al., 1995). As an example, Fig. 1-8B illustrates the H_2O_2 content in the wild type, *vtc1-1* and *npr1-1* single mutants and *vtc1-1 npr1-1* double mutants. All genotypes responded to *P. syringae* infection with an increase in H_2O_2 up to 48 h after inoculation. This increase was most pronounced in the wild type, which had even higher H_2O_2 levels 72 h post infection, whereas no further increase was observed in *vtc1-1*. H_2O_2 levels decreased in both *npr1-1* and *vtc1-1 npr1-1* mutants 72 h after inoculation (Fig. 1-8B) when peak bacterial density was reached in these mutants (Fig. 1-8A), which may be a result of demolished leaf integrity due to *P. syringae* infection.

Compared to mock-inoculated controls (Fig. 1-8C, D) and despite the SA biosynthesis or signaling defect, single and double mutants were able to induce free SA (Fig. 1-8E) and SAG (Fig. 1-8F) to some extent 72 h post inoculation. The difference in SAG content between the wild type and *vtc1-1* was less pronounced 72 h after infection, because the wild type induces SA accumulation. Large differences were observed in the SAG content in *npr1-1* single and *vtc1-1 npr1-1* double mutants, which contained approximately five times higher SAG levels than the wild type (Fig. 1-8F).

In summary, these data suggest that the activity of PAD4, EDS5, and NPR1 is required for the accumulation of SA and enhanced resistance against *P. syringae* in *vtc1-1*. To further investigate the intriguing role of NPR1 in the accumulation of H_2O_2 and SA, we performed a developmental analysis of the H_2O_2 and SA content in *vtc1-1 npr1-1* double mutants. Furthermore, to establish whether H_2O_2 functions upstream of SA and thus promotes the constitutive accumulation of SA in *vtc1-1*, we tested whether the accumulation of H_2O_2 and SA is attenuated when AA levels are increased artificially or *in vivo* in a *vtc1-1* suppressor mutant.

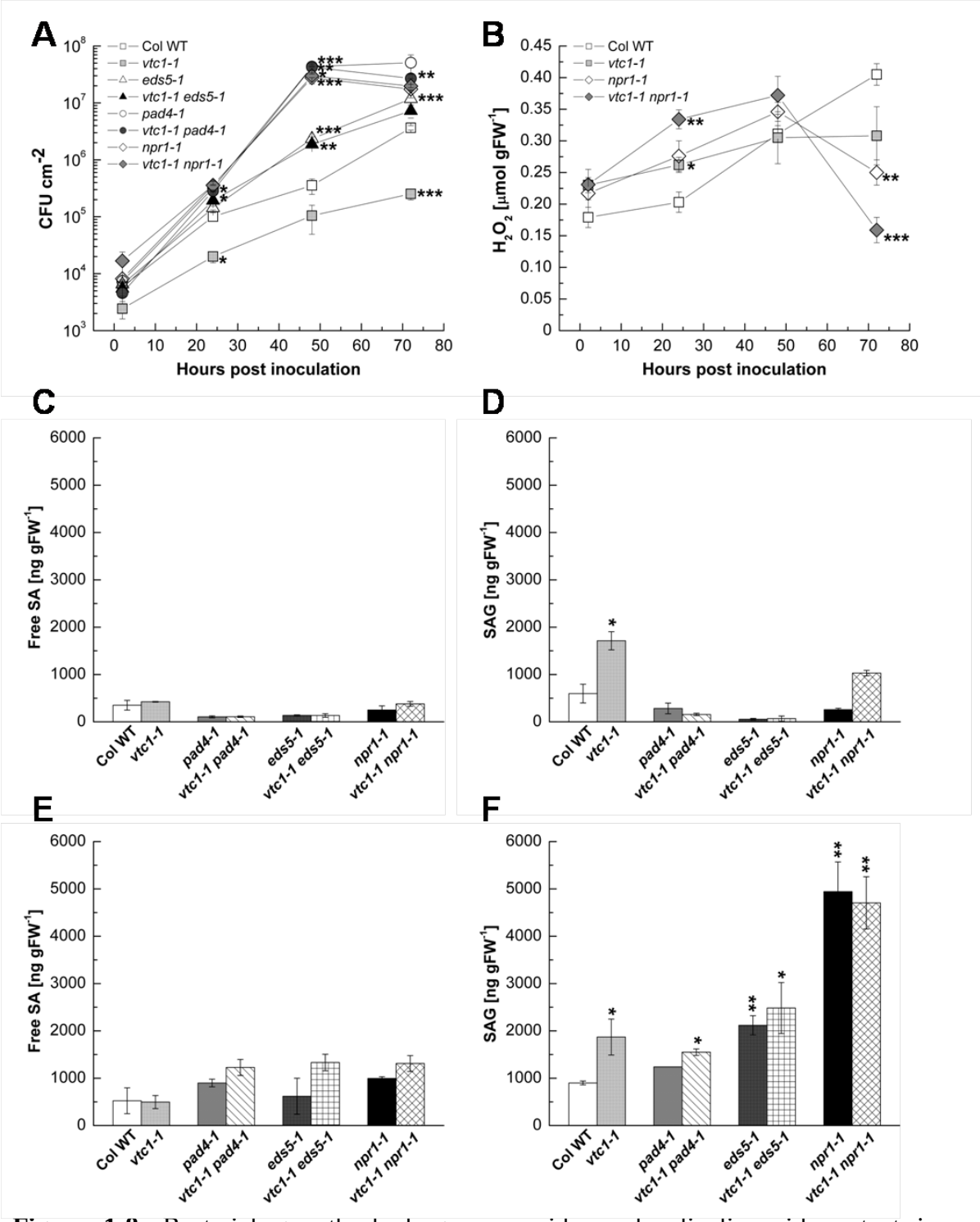

Figure 1-8: Bacterial growth, hydrogen peroxide, and salicylic acid content in Columbia-0 wild-type (Col WT), *vtc1-1*, *pad4-1*, *eds5-1*, and *npr1-1* single mutants and *vtc1-1 pad4-1*, *vtc1-1 eds5-1* and *vtc1-1 npr1-1* double mutant plants.
(A) Bacterial growth curve of *P. syringae*. Three-week-old plants were inoculated by dipping using a bacterial suspension of 10^7 CFU ml^{-1}. Leaf discs were collected at the indicated time points. (B) Hydrogen peroxide (H_2O_2) content based on g fresh weight (FW) throughout the infection process. (C) Free salicylic (SA) and (D) SA glucoside (SAG) content based on gram FW in wild-type and mutant plants in mock-infected plants 72 h after the start of infection. (E) SA and (F) SAG content based on gram FW in wild-type and mutant plants 72 h after *P. syringae* inoculation. Results depict means ± SE of three to four independent plants per genotype. Asterisks indicate significant differences between the wild type and mutants. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. (After Mukherjee et al., 2010)

1.3.2.3 The presence of the *npr1-1* mutation in *vtc1-1 npr1-1* double mutants suppresses H_2O_2 and SA accumulation in the absence of pathogen infection

To investigate whether SA accumulation is suppressed in *vtc1-1 npr1-1* because of suppressed H_2O_2 levels and/or because of alterations throughout development, we analyzed the H_2O_2 and SA contents in the wild type, *vtc1-1*, *npr1-1*, and *vtc1-1 npr1-1* mutants when plants were two, three and four weeks old.

As expected and as reported previously (Kotchoni et al., 2009), H_2O_2 levels increased throughout development in all genotypes. Although H_2O_2 levels were slightly but significantly higher in *vtc1-1 npr1-1* double mutants compared to the wild type in two- and three-week-old and unchanged in four-week-old plants, H_2O_2 content was significantly lower in the double mutant when compared to *vtc1-1* when plants were three and four weeks old (Fig. 1-9A). While the free SA content was largely similar among all genotypes at the three developmental stages tested (Fig. 1-9B), the SAG content was approximately three to six times higher in *vtc1-1* mutants than in the wild type at all developmental stages (Fig. 1-9C). Generally, SA content decreased in all genotypes when plants were four weeks old. The SAG content was largely unchanged in the wild type and *vtc1-1 npr1-1* mutants, but decreased in *vtc1-1* (Fig. 1-9C). Thus, the decline in SAG content in *vtc1-1 npr1-1* compared to *vtc1-1* single mutants was most pronounced in two-week-old plants. Note, however, that this change did not correlate with an altered H_2O_2 content at this developmental stage.

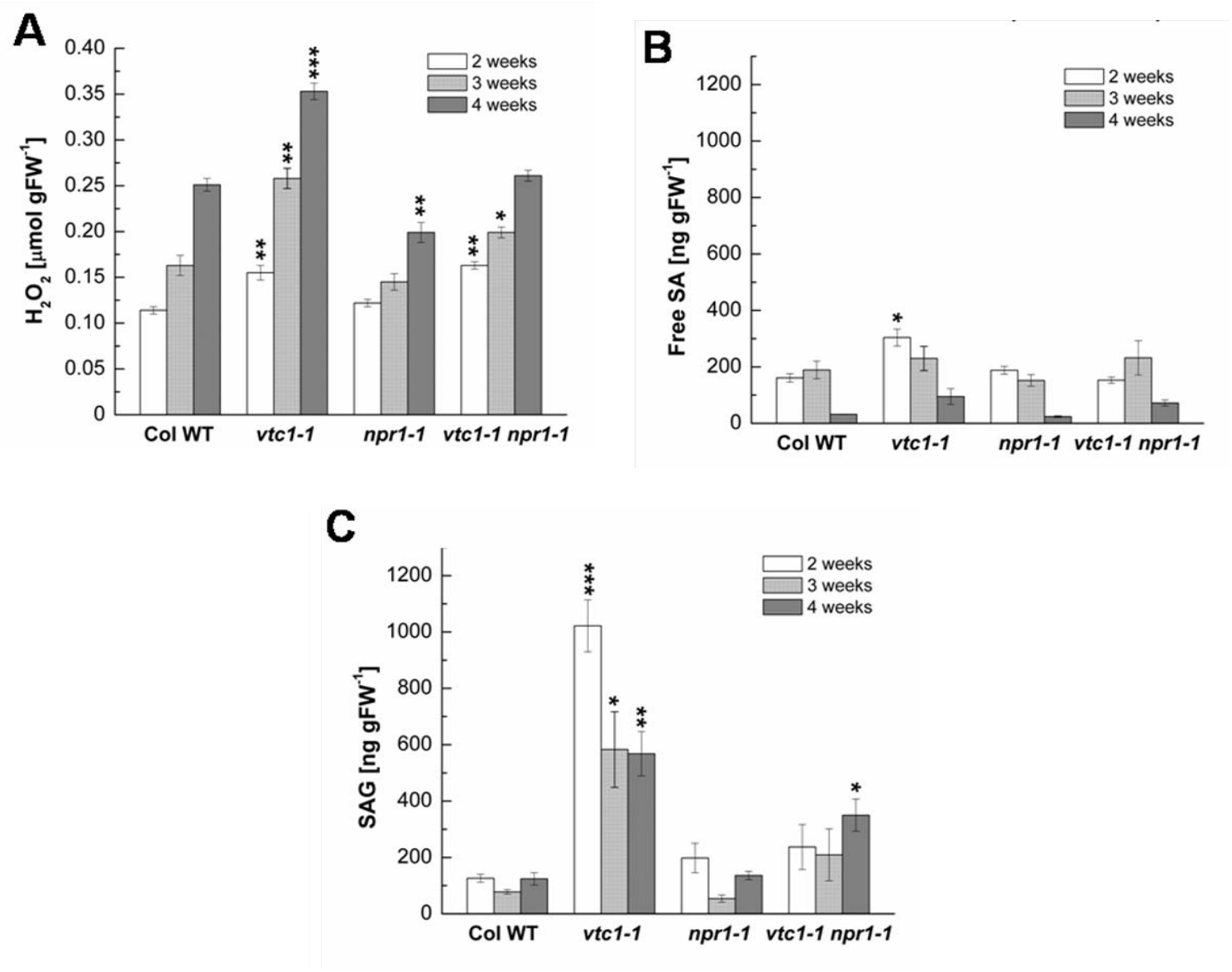

Figure 1-9: Hydrogen peroxide and salicylic acid content throughout development in whole rosettes of the Columbia-0 wild-type (Col WT), *vtc1-1* and *npr1-1* single mutants and *vtc1-1 npr1-1* double mutants.
(A) Hydrogen peroxide (H_2O_2) content based on gram fresh weight (FW). (B) Free salicylic (SA), and (C) SA glucoside (SAG), per gram FW. Results depict means ± SE of three to four independent plants per genotype. Asterisks indicate significant differences between the wild type and mutants. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.(After Mukherjee et al., 2010)

1.3.2.4. Higher ascorbic acid pool size decreases H_2O_2 and salicylic acid content

Ascorbic acid content was artificially increased by feeding the AA precursor L-galactose (L-Gal) to wild-type and *vtc1-1* mutant plants. If H_2O_2 induces SA accumulation, an increase in AA pool size should result in a decrease in H_2O_2 and SA levels.

L-Galactose spraying doubled the total AA content in the wild type and complemented the AA-deficiency phenotype in *vtc1-1* compared to the water-sprayed control plants (Fig. 1-10A). The H_2O_2 content was similar in both genotypes after L-Gal treatment and was significantly lower in both the wild type and *vtc1-1* compared to plants sprayed with water (Fig. 1-10B). While no significant change after L-Gal treatment was observed in the SA content in either genotype, the decreased H_2O_2 content in *vtc1-1* correlated with a decrease in SAG content. However, a similar trend was not observed in the wild type (Fig. 1-10C).

Further support for a role of H_2O_2 in affecting SA biosynthesis/accumulation comes from the analysis of the recently identified partial suppressor mutant *vtc1-1 fca-1* (Kotchoni et al., 2009). In addition to the *vtc1-1* mutation, the *vtc1-1 fca-1* double mutant contains a mutation in the autonomous flowering pathway gene *FCA*. The double mutant has approximately 75% of the Col/L*er* wild-type AA content, whereas in comparison to *vtc1-1*, the AA content is twice as high (Fig. 1-11A). Although the mechanism leading to the partially rescued AA content is presently unknown, we expected that H_2O_2 levels would be lower in *vtc1-1 fca-1* double mutants. The H_2O_2 content decreased to almost Col/L*er* wild-type levels in the double mutant (Fig. 1-11B). Thus, if H_2O_2 acts upstream of SA, we predicted that the *vtc1-1 fca-1* double suppressor mutant contains wild-type levels of SA. This is indeed the case (Fig. 1-11C).

In sum, the results presented in Figures 1-10 and 1-11 suggest that low levels of AA contribute to the formation of H_2O_2, resulting in the promotion of SA biosynthesis and/or accumulation in *vtc1-1*.

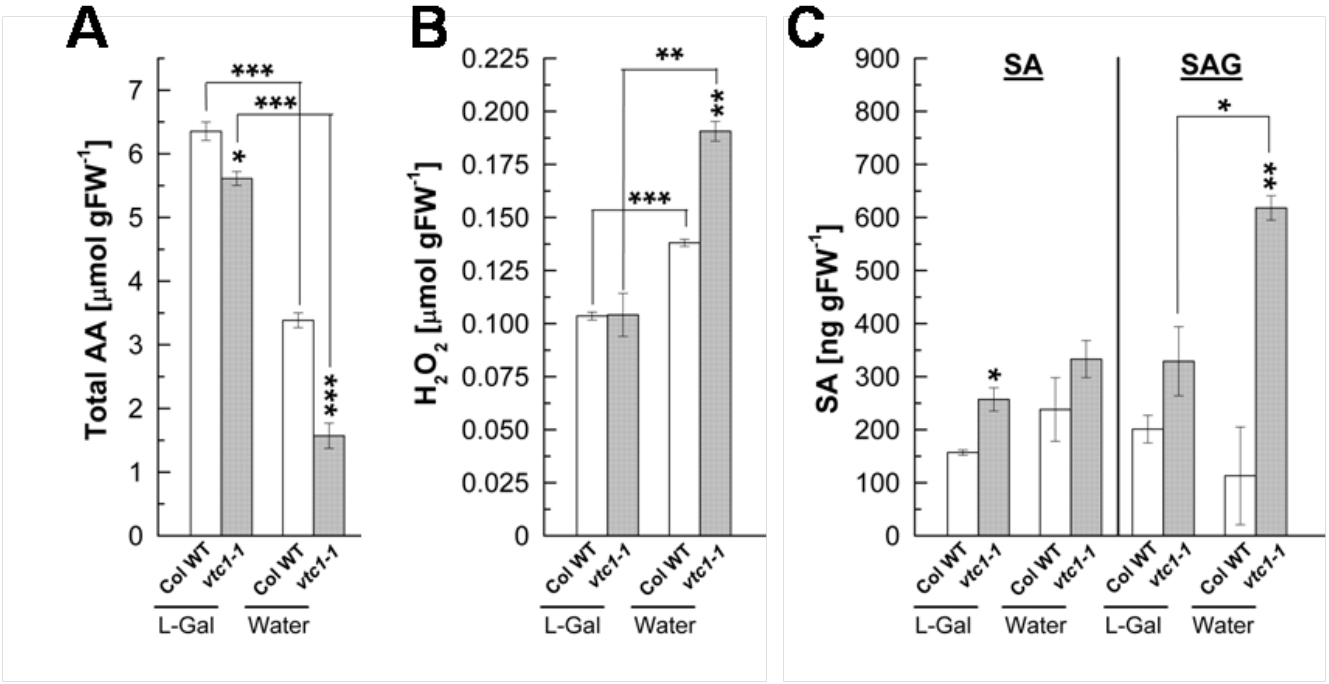

Figure 1-10: Effect of L-galactose on ascorbic acid, hydrogen peroxide and salicylic acid content in three-week-old plants of the Columbia-0 wild-type (Col WT) and *vtc1-1* mutants.

(A) Total ascorbic acid (AA) content based on gram fresh weight (FW) in L-galactose (L-Gal)- and water-treated plants. (B) Hydrogen peroxide (H_2O_2) content. (C) Free salicylic (SA), SA glucoside (SAG), and total SA content in the wild type and *vtc1-1* mutants treated with water or L-Gal. Results depict means ± SE of three to six independent plants per genotype. Asterisks indicate significant differences between the wild type and *vtc1-1* or between treatments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. (Mukherjee et al., 2010)

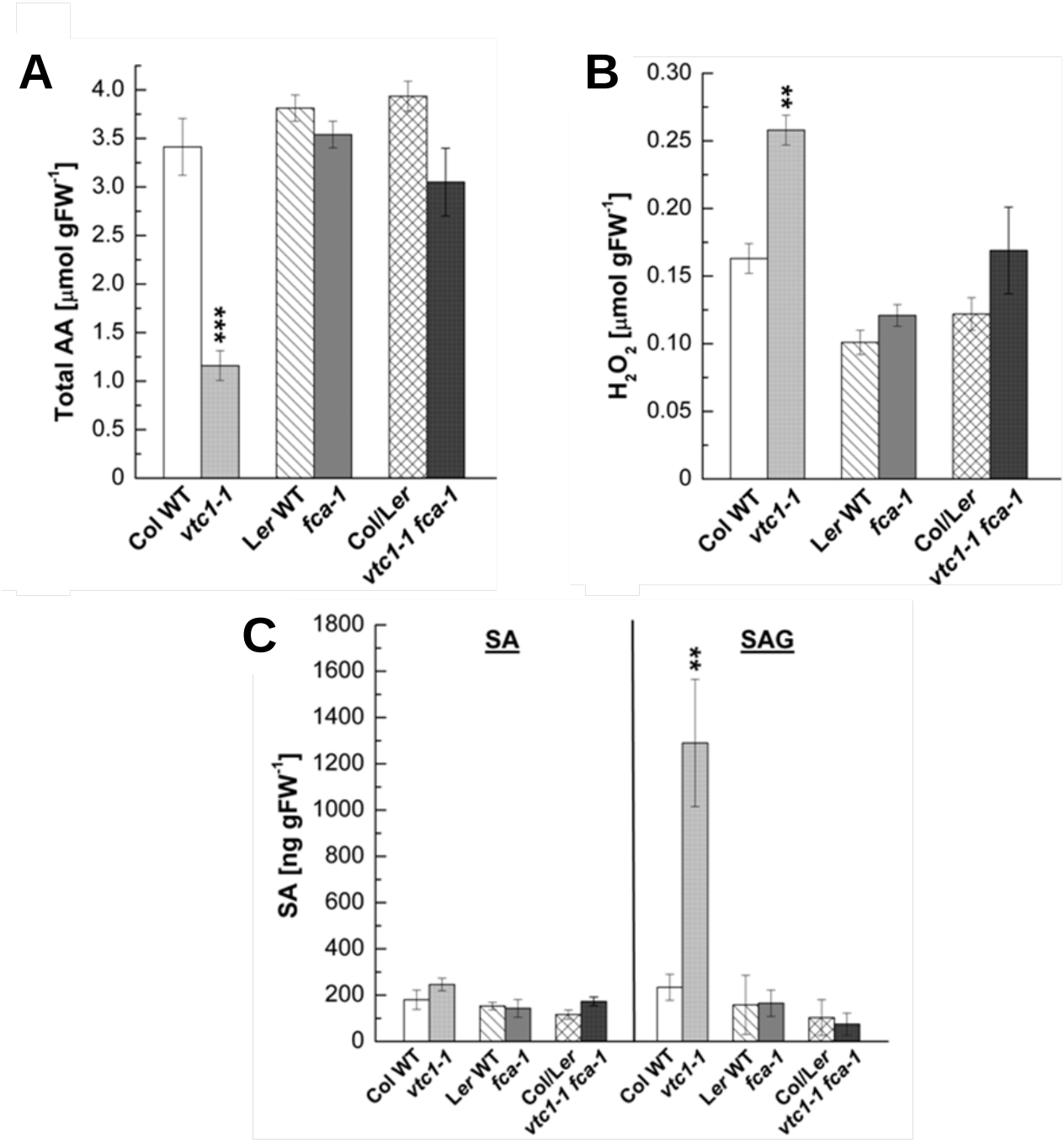

Figure 1-11: Ascorbic acid, hydrogen peroxide and salicylic acid content in the *vtc1-1 fca-1* partial suppressor mutant.
(A) Total ascorbic acid (AA) content based on gram fresh weight (FW) in Columbia-0 wild type (Col WT), *vtc1-1* (Col-0 background), Landsberg *erecta* wild type (L*er* WT), the autonomous flowering pathway mutant *fca-1* (L*er* background), a Col/L*er* wild-type cross, and *vtc1-1 fca-1* double mutants (Col/L*er* background). (B) Hydrogen peroxide (H_2O_2) content in the wild types, single and double mutants. (C) Free salicylic (SA) and SA glucoside (SAG) content in the wild types, single and double mutants. Results depict means ± SE of three to four independent plants per genotype. Asterisks indicate significant differences between the wild type and mutant. ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. (After Mukherjee et al., 2010)

1.4 DISCUSSION

1.4.1. Ascorbic acid deficiency causes early flowering that depends on the photoperiodic and autonomous flowering pathways

Earlier publications reported contrasting flowering and senescence phenotypes of the *vtc1* and *vtc2* mutants, exhibiting delayed and early flowering/senescence under SD (Pastori et al., 2003; Pavet et al., 2005) and LD (Barth et al., 2004; Conklin and Barth, 2004), respectively. Kotchoni et al. (2009) reported that all four *vtc* mutants, except *vtc2-1*, exhibit early flowering phenotype under both conditions, suggesting that AA deficiency causes this phenomenon irrespective of the photoperiod. Further investigation of *vtc* mutants that were not backcrossed to the wild type suggested that AA deficiency correlates with early flowering (Kotchoni et al., 2009). This is in contrast to findings by Pastori et al. (2003) and Pavet et al. (2005). The reason for the observed contrasting flowering phenotypes is presently unclear. Growth conditions, including nutrient supply and light intensity, may alter flowering time and could account for the differences in the time of flowering. For example, the lower light intensity used in experiments reported here (160 µmol photons m^{-2} s^{-1}) under both SD and LD conditions compared to the higher one used by the Pastori and Pavet groups (250 µmol photons m^{-2} s^{-1}) under SD could differentially affect AA pool size and redox status. Higher light intensities increase leaf ascorbate concentration (Smirnoff, 2000; Bartoli et al., 2006; Dowdle et al., 2007), whereas changes in photoperiods only affect AA pool size slightly or not at all (Queval et al., 2007; Kotchoni et al., 2009). Thus, one would expect delayed flowering in plants grown at higher light intensities. It would hence be worthwhile to investigate the flowering time of *vtc* mutants grown under different light intensities.

Ascorbic acid influences early flowering in the *vtc* mutants through a mechanism that is largely independent of its antioxidant property. Low levels of AA, as is the case in the *vtc* mutants (Conklin et al., 2000; Kotchoni et al., 2009), would be expected to result in elevated levels of ROS. The *vtc* mutants were shown to contain heightened H_2O_2 levels (Kotchoni et al., 2009), suggesting that they are suffering some oxidative stress under optimal growth conditions, ultimately resulting in early flowering/senescence. However, the following arguments do not support this hypothesis. First, the *vtc* mutants were shown

to suffer from more oxidative stress under LD than under SD, but the flowering phenotype did not correlate with the endogenous H_2O_2 levels. The *vtc* mutants flowered before the wild type under both SD and LD. Second, in comparison to the wild type, the differences in leaf number produced in the *vtc* mutants were similar under SD and LD conditions (Kotchoni et al., 2009). Third, *Arabidopsis* mutants lacking cytosolic ascorbate peroxidase and exhibiting constitutive accumulation of H_2O_2 under optimal growth conditions show delayed flowering under LD and constant light (Pnueli et al., 2003). It should be mentioned, however, that some double mutants deficient in cytosolic and thylakoid ascorbate peroxidase suffering oxidative stress exhibited early flowering (Miller et al., 2007). Finally, double mutants of *vtc1-1* and flowering time mutants are AA-deficient (Fig 1-6A), but have a delayed flowering phenotype irrespective of the photoperiod (Fig 1-6B and C).

1.4.2. Ascorbic acid deficiency induces priming, allowing for a more rapid induction of SA-mediated defense signaling

Evidence reported here suggests that AA-deficiency causes constitutive priming. Low levels of AA lead to increased SA levels, higher mRNA levels of *PR* genes, and enhanced resistance to *P. syringae*. The *Arabidopsis vtc* mutants resemble an intermediate phenotype between the constitutively primed *edr1*, *mpk4*, *cpr1* and *cpr5* mutants. While *PR1* transcripts were hardly detected in *edr1* in the absence of pathogens (Frye and Innes, 1998; Frye et al., 2001; van Hulten et al., 2006), *mpk4*, *cpr1* and *cpr5* exhibit constitutively high mRNA levels of *PR1* (Petersen et al., 2000; Wildermuth et al., 2001; van Hulten et al., 2006). Elevated *PR1* transcripts were observed in *vtc* mutants (except for *vtc4-1*), although mRNA levels were not as strongly elevated as in *mpk4* and *cpr1* mutants (Fig. 1-7C). However, PR1 and PR5 protein levels were not constitutively higher in *vtc1-1* (Barth et al., 2004). Salicylic acid levels are much higher in *mpk4* and *cpr1* than in *vtc* mutants (Fig. 1-7D) and mRNA levels of *SID2* and *PAL1* are constitutively higher in *cpr1* mutants (Wildermuth et al., 2001; Kohler et al., 2002), which was not observed in *edr1-1* (Beckers et al., 2009) and *vtc1-1* mutants in the

absence of priming agents or pathogens (data not shown). Yet, upon *P. syringae* infection, *vtc* mutants exhibited varying degrees of disease resistance (Fig. 1-7E).

In addition to SA, the key SA-signaling regulator NPR1 (Ahn et al., 2007; Beckers et al., 2009) and MAP kinase genes, including *MPK3* and the functionally redundant *MPK6* (Beckers et al., 2009), are required for priming. Our analysis of mutants with defects in the AA biosynthetic gene *VTC1* and the SA biosynthetic genes *PAD4* and *EDS5* as well as the SA signaling gene *NPR1* demonstrated that the constitutively elevated SA content in the *vtc1-1* mutant requires PAD4 and EDS5, and to some extent also NPR1 (Figs. 1-7D, 1-9B). Accumulation of SA in the *vtc1-1*, *vtc2-1*, and *vtc3-1* mutants is determined by an increase in the conjugated form of SA, the SA-glucoside (Fig. 1-8D). It is possible that SA glycosyl transferase, an enzyme that converts free SA to SAG, is activated in these mutants in order to reduce the amount of active SA (Lee et al., 1995). We hypothesized that SA is synthesized via the isochorismate synthase (ICS1 or SID2) and not the phenylalanine pathway, as it is known that the former is induced upon local and systemic pathogen infection (Wildermuth et al., 2001; Attaran et al., 2009). Although we did not generate double mutants between *vtc1-1* and *sid2* mutants, our data unequivocally show that constitutive accumulation of SA in *vtc1-1* occurs through the isochorismate pathway. This is supported by the fact that the *PAD4* and *EDS5* genes are upstream of SID2 (Nawrath et al., 2002), whereas *NPR1-1* acts downstream of SID2 (Shah, 2003; Glazebrook, 2005). Hence, priming in *vtc1-1*, and most likely in *vtc2-1* and *vtc3-1*, is dependent on SA biosynthesis mediated by PAD4, EDS5, and SID2, as well as NPR1, because *pad4-1*, *eds5-1*, and *npr1-1* mutations suppress SA accumulation and resistance in *vtc1-1* (Fig. 1-8A).

Assessment of transcript levels of *PAL1*, *SID2*, *MKK2*, *MPK3*, *MPK4*, *MPK6*, and *EDR1* genes that have been suggested to play a role in priming and whose expression is altered in constitutively primed plants, did not reveal a significant upregulation of these genes in *vtc1-1* in the absence of pathogen challenge (Mukherjee et al., unpublished results). Therefore, we suggest that priming in *vtc* mutants is mediated through a somewhat different signaling pathway. The enhanced disease resistance in *vtc* mutants may reflect an overlap between SA-induced defense responses and the genetic control of senescence. A similar mechanism has been suggested for the *edr1-1* mutant (Frye et al.,

2001; Tang and Innes, 2002). This is supported by the following facts: (i) *vtc1-1* enters senescence prematurely (Barth et al., 2004); (ii) several defense genes are induced during leaf senescence, including *PR1* (Hanfrey et al., 1996; Morris et al., 2000); and (iii) the senescence process is partially regulated by SA, as *Arabidopsis* mutants defective in SA signaling, such as *npr1-1*, *pad4*, and NahG transgenic plants, exhibit delayed yellowing and reduced necrosis (Morris et al., 2000).

Our data suggest that *vtc1-1*, *vtc2-1*, and *vtc3-1* can induce defense against *P. syringae* more rapidly through SA-dependent upregulation of *PR* genes. This, however, may not be the case in *vtc4-1*, which responds more subtly to *P. syringae* infection, not allowing us a simple conclusion as to what defense mechanisms are activated in this mutant (Fig. 1-6E). The *vtc4-1* mutant, in addition to *vtc1-1* and *vtc2-1*, has significantly increased ionically-bound peroxidase activity, suggesting that these mutants have higher cell wall peroxidase activity (Brady and Fry, 1997; Colville and Smirnoff, 2008). Cell wall peroxidase activity, which determines cell wall structure and mechanical properties through peroxidative cross-linking of cell wall proteins and polysaccharides (Brady and Fry, 1997), may contribute to cell wall strengthening in response to pathogens (Lamb and Dixon, 1997). Cell wall peroxidase can also generate H_2O_2, as was demonstrated in antisense plants with downregulated peroxidase, resulting in decreased apoplastic H_2O_2 and increased sensitivity to virulent and avirulent pathogens (Bindschedler et al., 2006). Therefore, altered cell wall composition and the induction of H_2O_2 after pathogen infection may contribute to the induction of defense responses in *vtc4-1*. Furthermore, it was demonstrated recently that *VTC4* is a bifunctional enzyme that not only affects AA but also myoinositol biosynthesis (Torabinejad et al., 2009). Myoinositol is incorporated into many crucial cellular compounds, such as phosphatidylinositol phosphate and myoinositol phosphate that are involved in signal transduction (Boss et al., 2006). Therefore, it is possible that other signals are important for priming in *vtc4-1*. This is supported by reports suggesting that establishment of SAR does not always correlate with an accumulation of SA and the mobile signal methyl salicylate (Cameron et al., 1999; Attaran et al., 2009). Furthermore, a study by Ahn et al. (2007) indicated that strong and long-lasting *PR1* transcription by treatment with vitamin B1, which induces priming, is not crucial for the induction of SAR (Ahn et al., 2007).

Finally, pathogen defense in the absence of *PR1* expression was reported in wheat (Gorlach et al., 1996) and barley (Jarosch et al., 2003) treated with the priming-inducing agents BTH, an SA functional analog, as well as in tobacco and rice treated with brassinolide (Nakashita et al., 2003).

1.4.3. Ascorbic acid deficiency-induced priming requires NPR1 and H_2O_2 in *vtc1-1*

As indicated above, NPR1 is required for priming in *vtc1-1*. This is not surprising, because the *npr1* mutant is priming-deficient (Kohler et al., 2002; Beckers et al., 2009). However, our data suggest a more complex function of NPR1 in regulating enhanced disease resistance in *vtc* mutants.

Accumulation of SA is higher in pathogen-infected *npr1* mutants than in pathogen-inoculated wild-type plants (Fig. 1-8F; Delaney et al., 1995; Shah et al., 1997; Attaran et al., 2009). This suggests that NPR1 plays a role in responding to elevated SA levels and controlling SA content. Our results support this function. Upon *P. syringae* infection, *vtc1-1 npr1-1* double mutants contain substantially higher SAG levels than the wild type and *vtc1-1* single mutants (Fig. 1-8F). Because of the genetic defect in NPR1, the protein does not function to induce defense genes in *npr1-1* single and *vtc1-1 npr1-1* double mutants. Thus, despite the accumulation of SA upon pathogen challenge, disease cannot be prevented in these mutants. This further supports the essential function of NPR1 in enhanced defense responses in *vtc1-1* and is consistent with what has been reported previously (Pavet et al., 2005). A more difficult question to answer is why the SAG content is suppressed in *vtc1-1 npr1-1* double mutants in the absence of *P. syringae* (Figs. 1-7D, 1-8D, 1-9C).

It is known that defense activators and subsequent pathogen infection trigger the formation of ROS, acting as signaling molecules stimulating SAR (Tenhaken et al., 1995; Wojtaszek, 1997; Alvarez et al., 1998). Specifically, H_2O_2 induces defense activation and enhanced pathogen tolerance in tobacco (Chamnongpol et al., 1998) and is required for priming (Ahn et al., 2007). The priming-deficient *npr1* mutant fails to accumulate H_2O_2 after priming with thiamine (vitamin B1) and challenge with *P. syringae* DC 3000 (Ahn et al., 2007). Furthermore, H_2O_2 has been shown to induce SA biosynthesis in tobacco

(Leon et al., 1995; Neuenschwander et al., 1995). Our experiments revealed that SAG but not free SA levels are repressed in *vtc1-1 npr1-1* double mutants, particularly at early developmental stages (Figs. 1-7D, 1-9C). However, our data do not support a relationship between H_2O_2, SA content and the function of NPR1. While the *npr1-1* mutation suppresses H_2O_2 accumulation in *vtc1-1 npr1-1* mutants to some extent (Figs. 1-7B, 1-9A), H_2O_2 content does not correlate with the suppression in SAG content in uninfected *vtc1-1 npr1-1* double mutants (compare Fig. 1-9A, C). This suggests that alterations in the SAG content in *vtc1-1 npr1-1* double mutants are independent of H_2O_2. This is further supported by the fact that *npr1-1* and *vtc1-1 npr1-1* mutants have low levels of H_2O_2 72 h post *P. syringae* infection, whereas SA content is greatly elevated (compare Figs. 1-8B and 1-8F). The decrease in H_2O_2 content may simply be explained by a loss of leaf integrity in *npr1-1* and *vtc1-1 npr1-1*. However, one might then also expect to see a decrease in SA content at this point in the infection process. This, however, is not the case. It is not clear whether antioxidant enzymes are activated in *npr1-1* and *vtc1-1 npr1-1*, thus contributing to the decrease in H_2O_2 content.

We can only speculate as to how NPR1 functions in the regulation of SA content in *vtc1-1 npr1-1* double mutants. Clearly, NPR1 serves as a negative regulator of SA biosynthesis when plants are challenged with *P. syringae* (Delaney et al., 1995; Shah et al., 1997; Attaran et al., 2009). However, NPR1 is required for the constitutive accumulation of SA in uninfected *vtc1-1* (Figs. 1-7B, 1-9C). It has been suggested previously that SA and NPR1 play broader roles in cell fate control and that NPR1 promotes cell division and/or represses endoreduplication during leaf development (Vanacker et al., 2001). This seemingly dual role of NPR1 may be explained by recent findings that suggest a role of chromatin remodeling in priming of SA-responsive loci in SAR (van den Burg and Takken, 2009). These studies suggest that prolonged SA accumulation upon pathogen challenge leads to NPR1-dependent repression of SA synthesis in an HDA19 (histone deacetylase 19)-dependent manner. This would explain why *npr1-1* mutants accumulate SA upon pathogen inoculation (Fig. 1-8F). Yet, it does not explain why SA levels are suppressed in *vtc1-1* by *npr1-1* in the absence of *P. syringae*. A correlation between SA, glutathione (GSH), and H_2O_2 contents in plants has been reported. Constitutive pathogen resistance in the *cpr1* and *cpr5* mutants

correlated with higher SA, GSH, and H_2O_2 content (Mateo et al., 2006). Mateo and coworkers suggested that high SA is associated with high GSH levels. Therefore, one would expect a substantially higher GSH content in *vtc1-1*, which contains approximately two times more free SA and eight times more SAG than the wild type when plants are only two weeks old (Fig. 1-9B, C). However, Conklin and co-workers did not find that AA deficiency in *vtc1-1* affects accumulation of reduced or oxidized glutathione (Conklin et al., 1996). Likewise, Pavet and co-authors only reported an approximately 1.4-times higher GSH content in *vtc1-1* and no change in GSH and a rather lower GSH redox status in *vtc2-1* when compared to the wild type when plants were two weeks old (Pavet et al., 2005). Similar results were reported by Colville and Smirnoff (Colville and Smirnoff, 2008). Consequently, the GSH pool is only affected to a small degree in the *vtc* mutants. We therefore suggest that the high SA content and NPR1 signaling are independent of GSH in *vtc1-1*. Although a role of GSH in promoting NPR1 monomerization has been reported previously (Mou et al., 2003; Pavet et al., 2005), it is now known that GSH is not the direct reducing agent of NPR1 *in vivo* and that thioredoxins catalyze NPR1 oligomer-to-monomer formation (Tada et al., 2008).

Although the function of NPR1 in regulating SA content in *vtc* mutants requires further investigation, we established a role of H_2O_2 in the induction of SA when AA is low (Figs. 1-10, 1-11). Our L-Gal spray experiments and investigation of the *vtc1-1 fca-1* suppressor mutant support the fact that H_2O_2 acts upstream of SA and contributes to SA induction in *vtc1-1*. However, there may be additional factors that stimulate SA biosynthesis. Our data are in contrast to those of Pastori et al. (2003), who previously suggested that the induction of PR proteins and disease resistance in *vtc1-1* is independent of H_2O_2 and SA, because H_2O_2 content was found to be similar to the wild type (Veljovic-Jovanovic et al., 2001) and *PAL* mRNA levels were not upregulated. Perhaps the H_2O_2 assay used here is more sensitive than the method used by Vejovic-Jovanovic and Pastori et al. Also, Pastori and co-workers did not measure the SA content.

In conclusion, when foliar AA levels are 40% or less of the wild type, formation of H_2O_2 is induced, resulting in isochorismate synthase-dependent accumulation of SA that also requires the function of NPR1. This may allow AA-deficient plants to adjust

redox homeostasis, thereby allowing plants to optimize growth and development in response to environmental changes, including virulent pathogens.

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APPENDIX

The work presented in this section summarizes some first preliminary data that were collected as part of a collaborative project between the laboratories of Dr. Alan Sexstone (Principal Investigator) from the Division of Plant and Soil Sciences, Dr. Kristen Matak (co-Principal Investigator) from the Division of Animal and Nutritional Sciences at the Davis College of Agriculture Forestry and Consumer Science, and Dr. Carina Barth (co-Principal Investigator) from the Department of Biology at the Eberly College of Arts and Sciences at West Virginia University. The project was funded by a West Virginia University Program to Stimulate Competitive Research (PSCoR) grant entitled "Algal Photobioreactor for Carbon Dioxide Capture and Biodiesel Production". Several undergraduate and graduate students were or are currently involved in this project. The graduate students involved in this project include Ms. Mariana Farcas, a graduate student in Dr. Sexstone's laboratory, and myself. The data presented below were produced in the laboratory of Dr. Sexstone and represent preliminary data that will be repeated in Dr. Sexstone's lab, and will be shared with Ms. Farcas as part of her Thesis project. The results I will present in the following would not have been possible without the work of two very talented undergraduate students, Benjamin Sade and Tabitha Amendolara, who carried out the initial experiments in the summer of 2009 as part of a WVU SURE and NSF REU stipend, respectively. I am also very grateful for the initial help and training that I received from Mariana and Jared Wilmoth to carry out the experiments and their contributions to the project.

A.1 INTRODUCTION

A.1.1 CO_2 mitigation and its importance

The amount of carbon dioxide (CO_2) in nature has increased as a result of human activities (Song, 2006). Its concentration has increased from a pre-industrial value of about 280 ppm to 379 ppm in 2005, which corresponds to 0.028-0.0379 % CO_2 in air (http://www.ipcc.ch/.2007). With the present rate of burning fossil fuels and deforestation, atmospheric carbon dioxide rates are predicted to reach 1000-2000 ppm in

the next few centuries (Walker and Kasting, 1992). However, the increasing concentration of atmospheric CO_2 has also been correlated to the rise in mean global temperatures (http://unfccc.int/meetings/cop_13/item/4049.php). Several research studies are underway to control the increase in atmospheric CO_2. For example, the use of energy sources which are carbon-free or have a lower carbon content, such as hydrogen, would be an option. Alternatively, strategies to sequester CO_2 may be developed in order to effectively remove CO_2 from the atmosphere and to thus stabilize and ultimately limit its accumulation (Department of Energy 1999). The different natural methods of carbon sequestration and long-term storage of the captured anthropogenic CO_2 that have been studied are ocean sequestration, such as deep ocean injection, or increasing the amount of dissolved CO_2 in the ocean, terrestrial sequestration, storing CO_2 in various geological formations such as in oil and gas fields (Stevens, 2000), and saline aquifers (Nordbotten et al., 2005). However, these processes have inherent limitations. For example, a team of researchers studying the effect of adding iron to the phytoplankton bloom in the southern Atlantic Ocean found that although this process was effective in increasing the phytoplankton biomass as well as photosynthetic rate in the surface waters, the amount of CO_2 being exported downward from the atmosphere did not increase significantly (Boyd et al., 2000). In case of terrestrial sequestration, the flux of CO_2 in this ecosystem is undetermined (Halmann, 1999). Carbon sequestration may also be achieved through chemical processes, such as chemically transforming CO_2 into thermodynamically stable forms or bicarbonate brines (Rau and Caldeira, 1999; Caldeira and Rau, 2000). However, such technologies have to be both safe for the environment and cost effective. Finally, the use of biological systems that are capable of photosynthesis, a process in which CO_2 is taken up form the atmosphere and converted into biomass, has attracted great attention as a potential mechanism to sequester carbon. Fixation of CO_2 through the process of photosynthesis has the added advantage in that oxygen, soluble biopolymers, carbonate and bicarbonate and volatile organic compounds are being produced, thereby transferring CO_2 to the aqueous phase of the system (Marcus, 1997; Munoz et al., 2004; Jacob-Lopes et al., 2009). Microalgae have been suggested to be most useful for biological carbon sequestration, because they can easily adapt to extreme environments and they rapidly produce biomass through more efficient photosynthetic activity (Lembi, 1988).

A.1.2 Importance of using algae in CO_2 mitigation

Algae can utilize up to 8% of available sunlight, a photosynthetic efficiency significantly greater than that of terrestrial plants. Algae grow rapidly (1 to 3 doublings per day) to dense biomass concentrations in open ponds or closed photobioreactors (Sheehan, 1998). It has been reported that in oceans phytoplanktons are able to fix CO_2 into biomass at a rate of 50-100 gigatons carbon (Gt-C) per year which is about ten times higher than the rate at which terrestrial plants fix CO_2 (Yamasaki, 2003). It might therefore be possible to cultivate large biomass of microorganisms, such as microalgae, in large waterbodies enabling CO_2 capture. This process, although it seems to be a much better option for CO_2 remediation, may pose a threat to the ecosystem and marine organisms. Because sinking and decaying biomass can give off stronger greenhouse gases such as methane and nitrogen dioxide (Chishlom, 2001). Therefore, growing algae in photobiorecators emerges as a sustainable and feasible option for CO_2 capture.

Most algae are composed of 5-20% phospholipids on a cell dry weight (CDW) basis. Under stress, many algae alter their lipid production largely through an accumulation of neutral lipids (20-50% CDW). These lipids, mainly in the form of triacylglycerols (TAGs), accumulate as dense lipid bodies in the cell cytoplasm. TAGs can be readily extracted and reacted with simple alcohols to produce alkyl esters plus glycerol, a process known as transesterification (Hu et al., 2008). The alkyl esters can be used directly for biodiesel, and the glycerol can be converted to value-added products (Pyle et al., 2008). Biodiesel has performance properties similar to conventional diesel, but upon combustion produces less particulate matter, CO_2, hydrocarbons, and SO_x (http://www.unh.edu/p2/biodiesel/article_alge.htm). Current biodiesel production in the U.S. from soybean oil averages 50 U.S. gallons per acre. Potential yields from algal biomass are projected to be at least 6000 U.S. gallons per acre (Haag, 2007). Thus, algae have the potential to capture significant amounts of atmospheric carbon and can be used to produce significant quantities of environmentally friendly biodiesel to offset combustion of hydrocarbon-derived transportation fuels.

Several studies attempted to identify species of algae that could potentially be used for optimizing CO_2 capture from industrial effluent gases. Yun and coworkers studied the potential of using *Chlorella vulgaris* in the effective removal of CO_2 from

flue gas as well as removing ammonia from the industrial wastewater discharged from a steel plant. They adapted *C. vulgaris* to grow in a 5% (v/v) CO_2 and ammonia removal rates were estimated as 26 g CO_2 m^{-3} h^{-1} and 0.92 g NH_3 m^{-3} h^{-1}, respectively, when the alga was cultivated in wastewater supplemented with 46 g PO_4 m^{-3} without pH control at 15% (v/v) CO_2 (Yun et al., 1997). A similar study conducted by Keffer and Kleinheinz utilized *C. vulgaris* in a photobioreactor with a very small gas residence time to demonstrate and establish the rate of CO_2 removal from an elevated CO_2 airstream. The authors showed that the CO_2 removal per unit biomass was 5.63×10^7 g CO_2 / g algal culture, where the total volume of the aqueous culture was 2000 cm^3 (Keffer and Kleinheinz, 2002). A preliminary study conducted by Jeong and associates compared the CO_2 fixation rate in *Chlorella* sp. H84, *Chlorella* sp. A2, *Chlorella sorokoniana* UTEX 1230, *Chlorella vulgaris* and *Chlorella pyrenoidosa* to determine their most efficient use in an industrial BioScrubber system. Among the different species tested, *Chlorella vulgaris* showed the highest growth rate with an optical density of 1.17 after 5 days of growth under culture conditions that included 243 µg CO_2/mL, 10 mM ammonia, and 1 mM phosphate with an initial pH range of 7-8 (Jeong et al., 2003). Several species of Chlorella isolated from hot springs in Japan were also shown to grow in temperatures up to 42˚C and air containing 40% CO_2 (Sakai et al., 1995). Other studies, investigating the growth response of *Chlorella vulgaris* under varying conditions of temperature and CO_2 have provided useful information pertaining to optimum growth of this species. Experiments carried out by Chinnasamy and coworkers showed that *C. vulgaris* produced 20 times more biomass at 6% CO_2 concentration compared to ambient CO_2 (0.036%) and a temperature of 30˚C. It was observed that at 16% CO_2 biomass production values were comparable to those at ambient CO_2 conditions, and the values decreased with more increase in CO_2 concentration (Chinnasamy et al., 2009). Xia and Gao demonstrated that *Chlorella pyrenoidosa* exhibited much better growth at 186 µmol CO_2 /L over 21 µmol CO_2 /L, while *Chlamydomonas reinhardtii* did not show any significant difference in growth (Xia, 2005). *C. reinhardtii* has also been tested for growth in artificial media and wastewater from industries in a biocoil system under varying conditions of CO_2 and pH and it has been found that this species was able to achieve the highest growth rate at 33.33% (v/v) where it reached an OD_{680nm} of about 2.25. Higher

CO_2 concentrations of 66.66% (v/v) or 100% (v/v) had lethal effects on the cells (Kong et al., 2010). These characteristics make microalgae highly desirable tools to mitigate CO_2 from flue gases, which typically contain 15% of CO_2 as estimated by the International Panel on Climate Change (IPCC) (Sakai et al., 1995). Additionally, the ability of microalgae to tolerate high temperatures could potentially reduce the cost of cooling the high temperature flue gas released from industrial exhausts.

A.1.3. Specific objective of the study*:* To physiologically characterize the two green algal species, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, in respect to their nutrient, pH and light requirements for maximal growth under elevated CO_2 conditions to maximize fixation of CO_2.

A.3 MATERIALS AND METHODS

A.3.1. Stock cultures and chemicals

Culture slants of *Chlorella vulgaris* (UTEX 2714) and *Chlamydomonas reinhardtii* (CH UTEX 90) were obtained from the University of Texas at Austin. The growth medium used in these experiments was a Wright's cryptophytes (WC) Medium (Guillard and Lorenzen, 1972) containing $NaNO_3$ (85.01 mg/l), $CaCl_2$ x $2H_2O$ (36.76 mg/l), $MgSO_4$ x $7H_2O$ (36.97 mg/l), $NaHCO_3$ (12.60 mg/l), Na_2SiO_3 x $9H_2O$ (28.42 mg/l), K_2HPO_4 (8.71 mg/l) supplemented with either 500 mg/l glycylglycine (buffer for axenic cultures) or 500 mg/l Tris buffer (for xenic cultures), trace metal solution (1 ml/l) and vitamin solution (1 ml/l). The trace metal solution contained Na_2EDTA x $2H_2O$ (4.36 g/l), $FeCl_3$ x $6H_2O$ (3.15 g/l), $CuSO_4$ x $5H_2O$ (10 mg/l), $ZnSO_4$ x $7H_2O$ (22 mg/l), $CoCl_2$ x $6H_2O$ (10 mg/l), $MnCl_2$ x $4H_2O$ (180 mg/l), Na_2MoO_4 x $2H_2O$ (6mg/l), H_3BO_3 (1 g/l). The vitamin solution contained thiamine-HCl (vitamin B_1, 100 mg/l), biotin (vitamin H, 0.5 mg/l) and cyanocobalamin (vitamin B_{12}, 0.5 mg/l). The final pH of the medium was 7.8. The stock cultures were maintained in slants containing either TAP medium (Tris acid Phosphatase) or glycylglycine WC medium with agar (15 g/l).

The inocula for each experiment were prepared by adding 3 ml of growth medium to the slants containing the stock cultures. The OD was measured at 750 nm using a *Spectronic 20* spectrophotometer to make sure that the starting OD (0.122) was the same for each inoculum. The inoculum was then injected into either the serum bottles or culture test tubes.

A.3.2. Assaying pH conditions for algae growth

A batch culture was used to investigate optimal pH conditions for growth. Twelve vertically held 200 ml test tubes in a wooden rack of 1.8 m in length were used. Six test tubes held 100 mL each of WC medium ranging from pH 4 to pH 10. Two 460-watt metal halide lamps suspended 1.5 meters above the test tubes supplied light. A strip of aluminum foil placed at a 45º angle reflected light onto the test tubes. A pH series of 5, 6, 8, 9 and 10 that was not inoculated was used as a control. The pH and OD were measured every 24 h for a period of 5 days. The batch cultures received 12 hours of light and 12 hours of dark daily. Air (ambient CO_2 level = 0.03%) at 50 ml/min was bubbled through 10 ml serological pipettes to agitate the solution.

A.3.3. Measuring biomass production as a function of CO_2 concentration

Batch cultures were used to determine optimum biomass production as a function of CO_2 concentration. Six 160 ml glass serum bottles were mounted on mini stir plates. The bottles were filled with 100 mL glycylglycine WC medium and six were filled with 100 ml Tris WC medium, and sealed with solid stoppers. Two needles punctured the stopper to bubble gas through the medium and to provide an exit and sampling point respectively. Air (ambient CO_2 level = 0.03%) was piped into a manifold that incorporated a cylinder of CO_2. Fine metering valves in the gas manifold controlled the flow of 50 ml/min, measured with an AD1000 Intelligent Flowmeter (Agilent Technologies, Santa Clara, CA). The CO_2 concentrations were measured using a Gas Chromatograph Carle AGC Series 100 (Carle Instruments, Anaheim, CA) against a pre-existing standard. Light intensity was 100 µmol m^{-2} s^{-1} (12 h light/12 h dark) using two GE grow lights (General Electric, Fairfield, CT).

A.3.4. Measuring CO_2 uptake rates in a closed growth system

One hundred sixty milliliter serum bottles containing 80 ml algal suspension of *C. vulgaris* with an OD of 0.065 were used in this experiment. Each contained an equal starting OD and 12% CO_2. Bottles were intermittently shaken. Three milliliters of gas were sampled at each time point, and the amount of CO_2 was analyzed. Temperature (21˚C) and light (118 μmol m^{-2} s^{-1}) were kept constant. The experiment lasted for 47 hours. There were six individual readings, with three replicates each. Three milliliters of headspace gas were extracted, and CO_2 was analyzed with a Carle AGC Series 100 gas chromatograph (Hach Co., Loveland, CO) connected to a BD40 chart recorder (Kipp & Zonen, Inc., Bohemia, NY).

A.4 RESULTS

A.4.1. Identification of an optimal growth medium with a broad buffering capacity

The WC growth medium (supplemented with glycylglycine or Tris) was tested for both pH stability as well as maximum biomass production using both *C. vulgaris* and *C. reinhardtii* under ambient CO_2 conditions (Fig A-1). Fig A-2A shows the growth curve of *C. vulgaris* in glycylglycine WC medium. Except for the medium at pH 4, OD for all other pH values (5-10) increased up to 1. For *C. reinhardtii*, however, this was not the case. After five days, the OD ranged between 0.3-0.5 for all pH values (except for pH 5).

The change in pH of this buffer was also monitored for the entire growth period of both *C. vulgaris* and *C. reinhardtii*. This medium had good buffer capacity and maintained the pH between 7 and 8.8 (Fig A-2B and D), suggesting that this pH range provides optimal conditions for growth of both algal species.

Figure A-1: Setup for conducting experiments to identify optimal pH of growth media for *C. vulgaris* and *C. reinhardtii*.
200 ml test tubes were vertically held against a wooden rack and each tube was filled with 100 ml of growth medium. The mouths of the tubes were covered with foam stoppers through which an air line and a sampling line were inserted. The light source was placed directly overhead of the setup.

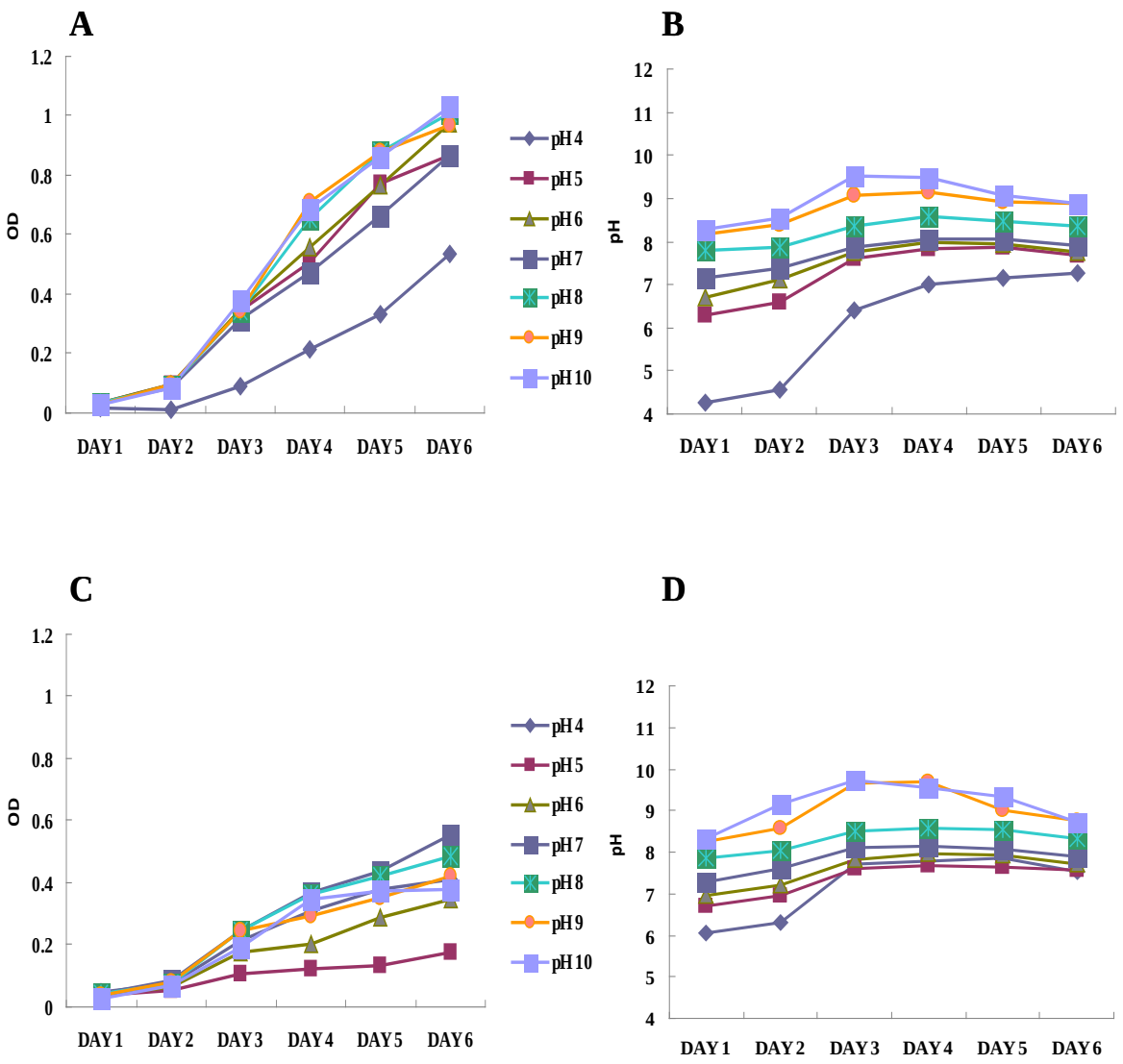


Figure A-2: Growth and pH change of *C. vulgaris* and *C. reinhardtii* grown in glycylglycine WC medium.
(A) Change in OD over time for *C. vulgaris* for a pH change of 4-10. (B) Change in pH over time for *C. vulgaris*. (C) Change in OD over time for *C. reinhardtii* for a pH change of 4-10. (D) Change in pH over time for *C. reinhardtii*.

A similar setup was used to evaluate the growth of these two algal species in the WC medium supplemented with Tris (Tris WC). *C. vulgaris* exhibited slightly better growth at pH 8 in this medium, reaching an OD of 0.735 after five days of growth (Fig A-3A). However, compared to the glycylglycine WC medium (see Fig A-2A), TRIS WC did not support growth of *C. vulgaris* to the extent it was seen in the glycylglycine WC medium. In case of *C. reinhardtii,* the maximum OD was even smaller (between 0.1 and 0.4) (Fig A-3C). However, the buffering capacity of the TRIS WC medium was similar to the glycylglycine WC medium for both algal species (Fig A-3B and A-3D).

Taken together, these results demonstrate that the WC medium supplemented with glycylglycine supported superior growth of algae, and that *C. vulgaris* displays a much higher growth rate over time than *C. reinhardtii.* This medium supported better growth for both species compared to previously tested TAP medium (BJ Sade, TA Amendolara and M Farcas, unpublished results).

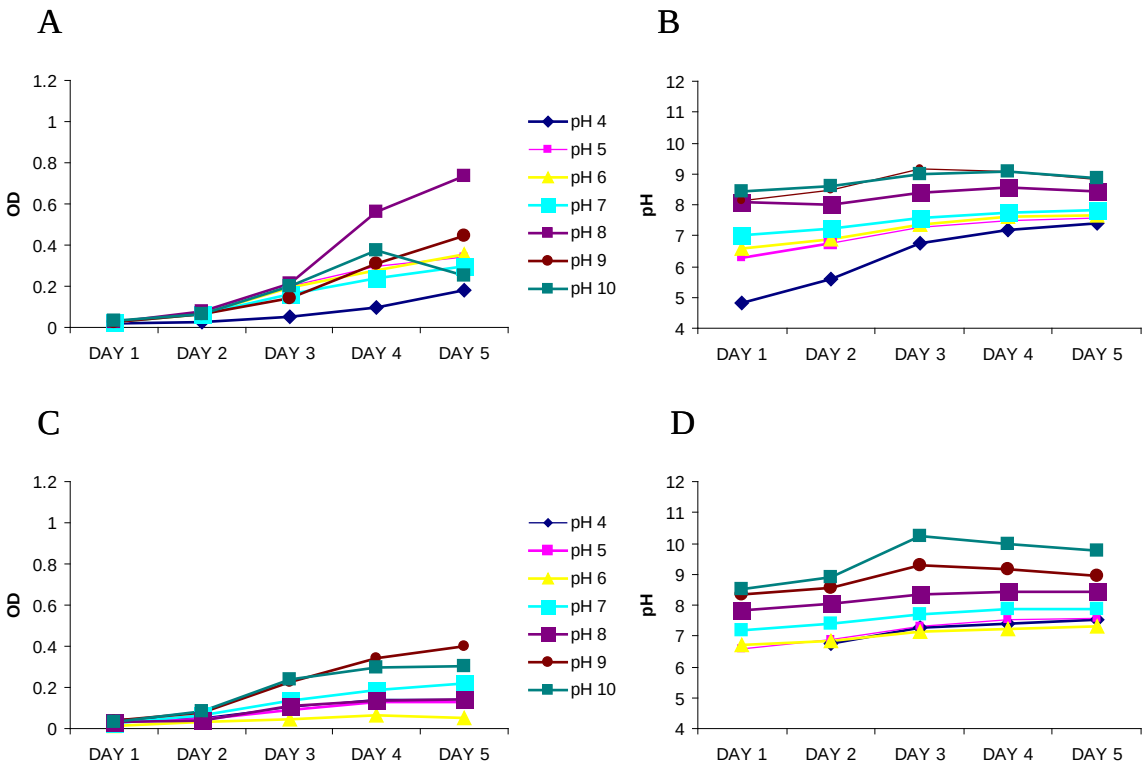


Figure A-3: Growth and pH change of *C. vulgaris* and *C. reinhardtii* in Tris WC medium.
(A) Change in OD with time for *C. vulgaris* for a pH range of 4-10. (B) Change in pH with time for *C. vulgaris*. (C) Change in OD with time for *C. reinhardtii* for a pH range of 4-10 and (D) Change in pH with time for *C. reinhardtii*.

A.4.2. Growth of *C. vulgaris* and *C. reinhardtii* in ambient (0.03%) and elevated CO_2 (12%) conditions in an open growth system

In the open growth system, CO_2 was constantly bubbled into serum bottles for a period of five days and algal growth was monitored by measuring the OD over time (Fig A-4). Alterations in the pH in Gly WC medium were also recorded in order to investigate the buffering capacity of the medium when higher CO_2 concentrations were injected into the growth medium. This was important to test because dissolved CO_2 will acidify the growth medium and could negatively affect growth. It was observed that under ambient CO_2 conditions, *C. vulgaris* produced more biomass than *C. reinhardtii*. Under 12% CO_2, growth of *C. vulgaris* increased substantially. However, this was not the case for *C. reinhardtii* (Fig A-5A).

The initial pH of the growth medium at the time of inoculation was maintained at 7.8. The pH remained within the range of 6-8 for all the above mentioned conditions. This suggests that this range of pH coupled with the strong buffering capacity of Gly WC medium allowed for optimal growth of *C. vulgaris* (Fig A-5B).

Figure A-4: Set up showing elevated CO_2 experimental design in serum bottles.

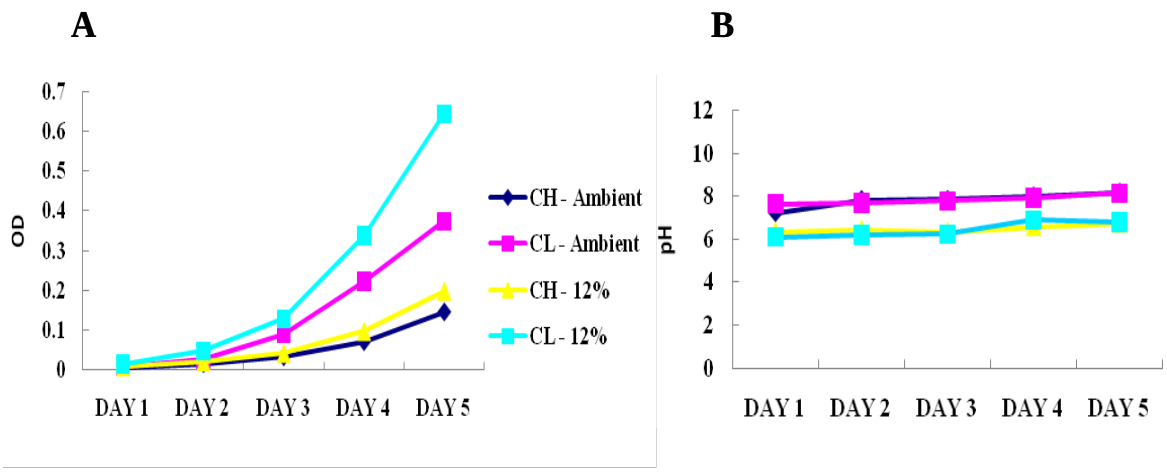


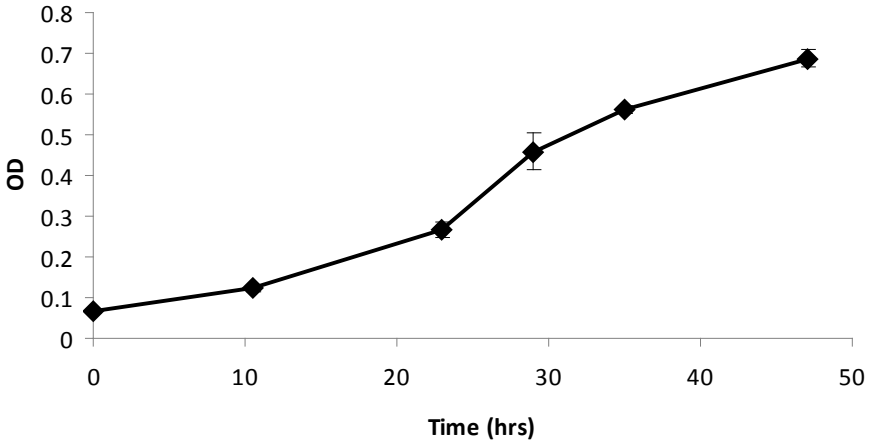
Figure A-5: Growth of *C. vulgaris* (CL) and *C. reinhardtii* (CH) under ambient and 12% CO_2 in glycylglycine WC medium.
(A) Change in OD over time. (B) Change in pH over time.

A.4.3. Growth of *C. vulgaris* in a closed growth system to determine CO_2 uptake from the atmosphere

The set up used for this experiment was essentially the same as the one used for the open system experiment (see Fig A-4) except that once the headspace of each serum bottle was filled up with 12% CO_2, the gas lines were disconnected and the system was not continuously aerated. The serum bottles were placed on magnetic stir plates to ensure constant agitation. Each serum bottle initially contained an OD of 0.065 *C. vulgaris* and 12% CO_2.

Fig A-6A shows the increase in biomass over time. The growth curves show a lag phase of about 10 hours before an increase in growth rate occurred. The growth rate correlated nicely with the decrease in CO_2 concentration (Fig A-6B). These data indicate the total amount of time required by *C. vulgaris* to effectively consume the headspace CO_2 provided in each serum bottle, which in this experiment was approximately 47 hours.

A



B

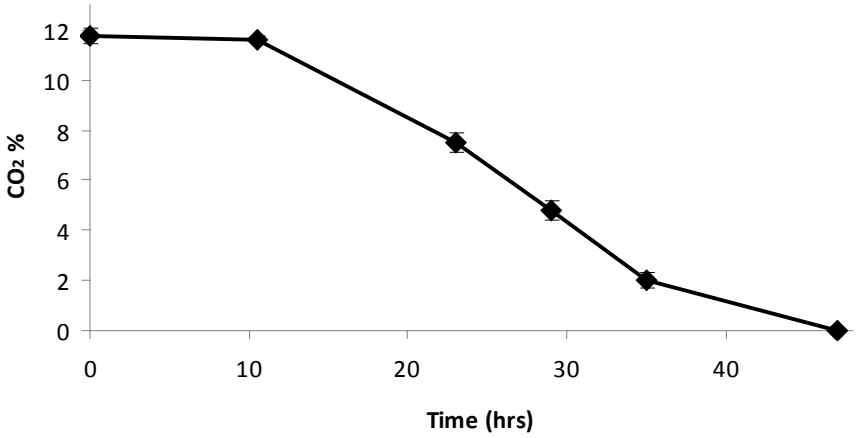


Figure A-6: CO_2 consumption in a closed system containing 12% CO_2 conditions. (A) Increase in biomass of *C. vulgaris* as a measure of OD change over time. (B) CO_2 consumption of *C. vulgaris* with respect to time of three replicates. Mean values ± SE of three independent replicates are shown.

A.5 DISCUSSION

The overall goal of this project was to identify optimal growth conditions in respect to growth medium, pH, and to compare growth rates of *C. vulgaris* and *C. reinhardtii* in an effort to mitigate CO_2 from environmental sources. The feasibility of the use of *C. vulgaris* as a potential candidate for CO_2 mitigation seems likely. Our results demonstrate that *C. vulgaris* proliferates better than *C. reinhardtii* using the various growth media tested in this study. One study specifically cited the use of *C. reinhardtii* as a source for biofuel production, because it is a well studied and documented algal species (Hu et al., 2008). However, this study demonstrated that it is worthwhile to investigate other algal species in regards to their growth. The data obtained for *C. vulgaris* are in accordance with results reported by Xia and Gao (Xia, 2005). These authors showed that *C. vulgaris* supported much better growth and biomass production under elevated CO_2 conditions (186 µmol /L CO_2) than *C. reinhardtii*.

Once algal growth has been optimized, strategies to enhance lipid production need to be developed. Studies have shown that by increasing carbon dioxide content, algal growth and lipid production increases (Hu et al., 2008). Although we tested higher CO_2 concentrations, it is unclear whether the increased growth also contributed to increased lipid production. Other factors that will also need to be investigated further are salinity of the medium, temperature, and limiting key nutrients. Investigating these factors will be important because it is well established that in algae exposed to stress including nutrient limitation or suboptimal temperatures, lipid production may be induced (Woertz et al., 2009).

In summary, our results show that among the two species of algae tested *C. vulgaris* exhibits maximum growth under conditions of optimal pH, light and increased CO_2. We have successfully standardized a growth medium (glycylglycine WC) that supports maximum growth of this alga, because it has a superior buffering capacity under increased concentrations of CO_2. However, whether or not this medium is suitable for growth of *C. vulgaris* in a photobioreactor remains to be determined. It is also important to note that this medium might not be the most cost-effective way to optimally grow *C. vulgaris* for the production of lipids. Future experiments will be directed towards understanding how the physiology of algal cells is affected by examining photosynthetic

activity under various light, CO_2, and nutrient conditions. Other experiments will investigate the effect of increased CO_2 on photosynthetic enzymes, overall carbon assimilation, and lipid production.

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